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# THE BIOLOGICAL BULLETIN

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# THE BIOLOGICAL BULLETIN

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## THE EXTERNAL DEVELOPMENT OF THE BANDED DOGFISH OR POFADDERHAAI HAPLOBLEPHARUS EDWARDSII (M. & H.)

CECIL VON BONDE

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### INTRODUCTION

This oviparous species of dogfish, previously known as *Scylliorhinus edwardsii* (Cuv.), is endemic to South African waters and has been recorded from Saldanha Bay, Table Bay, False Bay and the Agulhas Bank. The largest one on record measured 520 mm. This dogfish is fairly plentiful and specimens are on exhibition in the Sea Point Aquarium.

On November 5, 1942, a female was observed to lay two egg cases in one of the tanks of the Aquarium. One case was immediately removed and dissected to expose the egg, whilst the other was left in the tank as a control in the determination of the duration of incubation.

The egg was completely removed from the egg case and placed under observation in running sea water.

### THE FEMALE REPRODUCTIVE ORGANS

The only external sexual characters in the female are the pair of cloacal papillae (Plate I, c.p.), one situated on either side of the median line immediately posterior to the cloacal aperture. The papillae themselves are perforate, and the abdominal pore situated in the center of each papilla connects direct with the coelom. The actual function of these abdominal pores is not known and it is obvious that, in view of the highly specialized structure of the female genital system, they have lost the function of acting as apertures through which the ova leave the body.

A dissection (Plate I) revealed the fact that there was only a single median ovary (ov.) present which contained ova in various stages of development. The ovary is situated in the middle portion of the coelom and is relatively large. The paired oviducts are highly specialized both in structure and function. The oviducts meet medially in the anterior part of the coelom (f.t.o.). Each oviduct may be divided into four distinct portions as follows:

(1) The fallopian tube (Plates I and II, f.t.) is extremely narrow and thin-walled and is about a quarter of the length of the whole oviduct. Its internal surface is slightly convoluted longitudinally. This portion is followed by (2) the albumen gland (a.g.) which is about one-eighth of the total length of the oviduct and is very thick-walled and muscular. This gland secretes the albumen which sur-

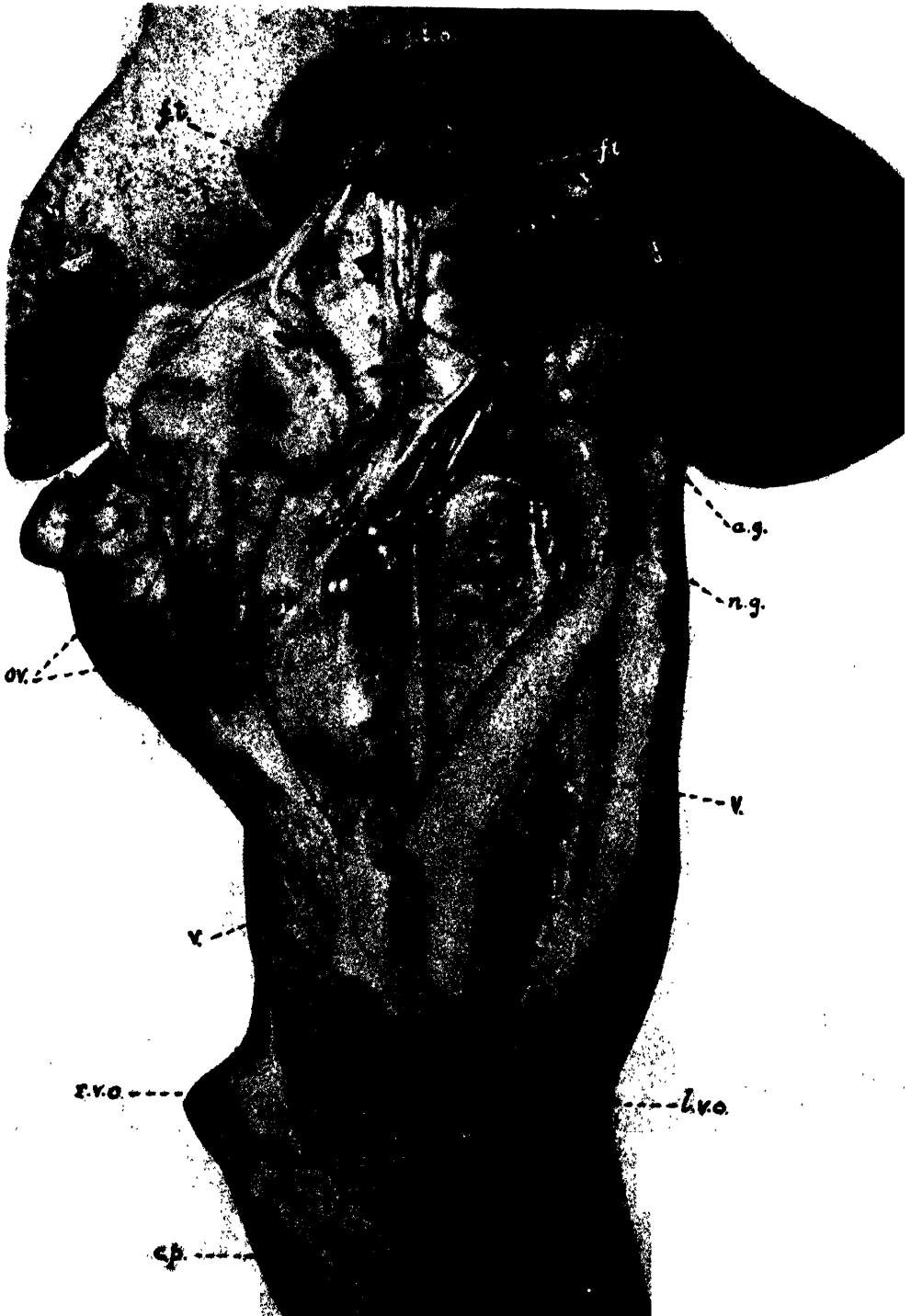
rounds the ovum, hence its internal surface is richly supplied with glands for this purpose. A transverse section of the albumen gland (Plate II, Fig. 3, l.a.g.) shows that the lumen is extremely narrow, the walls of the gland being completely apposed. Smith (1942, p. 703) in describing Bashford Dean's figure of the reproductive organs of an adult female *Heterodontus japonicus* states—"This drawing (my Text-figure 35) is not labelled, nor is it described in Dean's notes, and in the absence of the dissection some features are obscure. In the mid-line near the top of the figure, one readily notes the common abdominal opening of the oviducts. On the extreme right side of the figure the oviduct with its three divisions—oviduct proper, shell gland, and uterine portion—are easily identified." The part of the oviduct designated "shell gland" in the above quotation is very similar in external appearance to that of the present species, but as previously stated, this swollen portion functions as the albumen gland in the present species. There are no indications of a laminated structure in this gland as described by Gudger (1940, p. 550) for the nidamental gland of *Chlamydoselachus*. Immediately following the albumen gland is (3) the nidamental gland (n.g.) which is also about one-eighth of the total length of the oviduct and secretes the shell around the ovum. The interior surface of the nidamental gland is extremely well convoluted and is of a dark yellowish color. Widakowich (1907, p. 527) states—"Das Nidamentalorgan von *Acanthias* besteht aus einem cranial gelegenen Teile, der, wenn man nach Analogie mit *Scyllium* schliessen darf, Eiweiss erzeugt, aus einem mittleren, der, wie sich zeigen lässt, die Schale liefert, und aus einem untersten, wohl Schleim produzierenden Abschnitte." As the functions of the various parts of the oviduct of the present species, however, are distinctly diverse, it appears to be better to look upon each part as an independently functioning entity and parts two and three as here described may be looked upon as analogous to the cranial portion, and the central and caudal portions of the nidamental gland, respectively, as described by Widakowich. The last part (4) of the oviduct is the vagina (v.), occupying about one-half of the total length. This portion of the oviduct is designated the vagina in analogy to the use of this term in mammals in view of the fact that the intromittent organs (claspers) of the male are inserted through the vaginal orifices during copulation.

Each oviduct opens separately by a vaginal orifice (Plate II, l.v.o. and r.v.o.) into the cloaca. The vagina is well convoluted longitudinally to permit of distension both during copulation and during ovulation. In certain of the females dissected, parts of the mermaid's purse were present in the upper half of the vagina where it leaves the nidamental gland. These parts consisted principally of the anterior and posterior parts of the mermaid's purse complete with the tendrils (see Plate IV, Fig. 6).

#### PLATE I

Dissection showing the female reproductive organs of *Haploblepharus edwardssi* (M. & H.). a.g., albumen gland. c.p., cloacal papilla. e.g., external gill-filaments. f.t., fallopian tube. f.t.o., coelomic orifice of same. h., hooks of clasper. h', hooks normally covered by rhipidion. hy., hypopyle. l.a.g., lumen of albumen gland. l.v.o., left vaginal orifice. n.g., nidamental gland. ov., ovary. r., rhipidion. r.v.o., right vaginal orifice (opened). u., umbilicus with remains of cord. u.c., umbilical cord. v., vagina. v.b.v., vitelline blood vessel. y.s., yolk sac.

PLATE I



## THE MALE REPRODUCTIVE ORGANS

The morphology of the male reproductive system follows the general plan of all Elasmobranchs. The testes are paired and occupy the anterior half of the coelom. From the anterior end of each testis the vasa efferentia lead to the epididymus which is much coiled. The epididymus eventually widens out into the vas deferens which expands and joins its fellow in the median line posteriorly, opening into the cloaca by a single pore.

The secondary sexual characters of this species are very distinct and it is necessary to describe them in more detail. As in all male Elasmobranchs, the basal element of each pelvic fin (*basipterygium*) is prolonged to form a stout, backwardly-directed rod which is sharply demarcated from the remainder of the fin and specially modified to form an intromittent organ commonly known as the clasper or myxopterygium. In this species the clasper is a very highly differentiated organ which differs in many respects from that found in other Elasmobranchs. In transverse section the clasper is almost completely round in its proximal half, the internal cartilagenous skeleton forming a tube which is almost completely filled with a thick muscular substance through the center of which passes a duct. In the distal part the skeleton only occupies the ventral portion of the clasper, being continued right to the tip. The latero-dorsal wings of this portion of the clasper are formed of thickened skin, the outer wing being folded over the curved inner wing so as to form a canal which is a continuation of the duct in the proximal portion previously mentioned. This canal opens on the dorsal side of the posterior end of the clasper in the form of a hypopyle (Plate III, Fig. 4, hy.). Schmidt (1930) first noted the existence of hooks in the clasper of *Halaclurus torazama* which is a genus closely related to the present one and he noted that these hooks were approximately 100 in number. The clasper of the present species also shows approximately 100 hooks on the outer lateral wall of the clasper (h. and Fig. 5).

Schmidt states—"Considering now what purpose this arrangement can serve, one comes to the conviction that the hooks can be used only for fastening the clasper on the wall of the vaginal part of the oviduct of the female during the copulation. Probably, when the clasper is introduced, the borders of its gutter are turned out and all the hundred hooks of the row are imbedded in the oviducal wall, holding the clasper as anchors. If the other clasper is introduced simultaneously in the other oviduct, the male is held by 200 little anchors. This arrangement may be necessary, as the female of this shark is larger than the male, which is perhaps carried about by the female during a copulation that may continue for a very long time.

"This arrangement of the organs of copulation of the male seems to be unique not only among the fishes, but perhaps in the whole animal kingdom," but he makes no mention of the other series of hooks (h') which lie on the inner lateral wall of the clasper and which are covered by a rhipidion (r.), whose function is to spray the spermatozoa in all directions in a radiating manner, under the pressure exerted

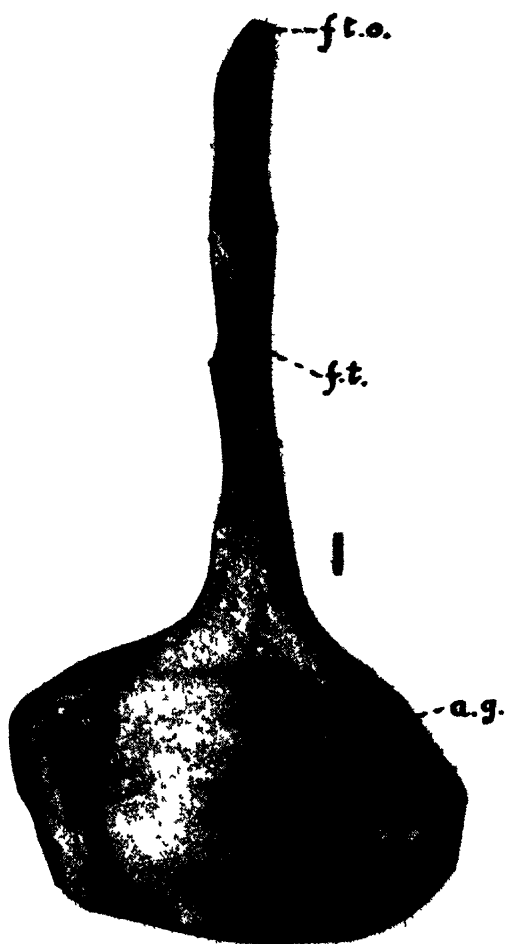
## PLATE II

The left oviduct opened medially to show the internal structure.

FIGURE 1. Longitudinal section through the fallopian tube and the albumen gland.

FIGURE 2. Longitudinal section through the nidamental gland and the vagina.

FIGURE 3. Sagittal section through the albumen gland.



3



2

by the water forcibly ejected by the siphon. The reason for this is obvious since fertilization takes place in the fallopian tubes and the spermatozoa, although motile, have to traverse the complete length of the vagina, nidamental gland, and the albumen gland to reach the ova in the anterior end of the fallopian tubes.

Leigh-Sharpe (1920) in describing the secondary sexual characters of *Scyllium catulus* mentions the presence of a siphon on the ventral surface of each pelvic fin. The present species also has such a siphon developed in the same position, although it is probably more analogous to the clasper gland mentioned by Leigh-Sharpe (op. cit., p. 260) than to the siphon. The whole of the clasper except the distal end is covered with dermal denticles. A deep dissection of the clasper showed that it is in no way innervated.

### DEVELOPMENT

In order to study the external development of this species a mermaid's purse was opened, the ovum removed from the purse and placed in a dish of running sea water. The albumen was completely removed so as to enable one to observe the development of the embryo.

The egg case or mermaid's purse (Plate IV, Fig. 6) is more or less typical of those formed by oviparous dogfishes. The anterior end is broad and almost as wide as the main body and has a straight edge where the two halves have partially coalesced. From each lateral end a long coiled filamentous process, which gradually becomes thinner towards the distal end, arises. Near the base of each of these tendrils the purse has a longitudinal slit-like aperture (s.) which leads to the interior. These apertures only appear on one surface of the egg case. Posteriorly the egg case tapers until it is little more than half as wide as the anterior end. At the lateral extremities of this sharper end two processes arise which are the bases of the tendrils. These posterior tendrils are much longer and thicker than the anterior ones at their points of origin, but, like the latter, they also become thinner towards their distal ends. It is this pointed end of the purse which first appears through the vaginal orifice when the egg case is laid. The function of the tendrils is to anchor the egg case securely to rocks or seaweed to prevent its being buffeted about by the waves. The egg case itself is formed from keratin secreted by the nidamental gland. Widakowich (1906) stated that the mermaid's purse of *Scyllium canicula* is formed by a large number of separate elements ("Platten") which later, on exposure to sea water, adhere closely to form a complete shell (see also Hobson (1930, p. 580)), but in *Haploblepharus edwardsii* there is no evidence of such a formation of the egg case which is here laid down completely in two halves, the dorsal and ventral parts, the edges of which coalesce.

A dissection of the mermaid's purse shows that it is a structure formed of two portions which may be designated the dorsal and ventral halves, each half being hollow, the edges being apposed and completely fused along their whole length except at the blunt anterior end where the apposition is purely temporary. It is

### PLATE III

FIGURE 4. Dorsal view of the left clasper with lateral wings of distal portion distended to show internal structure.  $\times 4$ .

FIGURE 5. A series of 40 hooks removed from the clasper.  $\times 10$ .



through this anterior end that the fish eventually escapes, hence the lack of fusion of these edges. The swollen central portion occupying about two-thirds of the internal space of the egg case, is filled by the relatively enormous yolk. Surrounding the yolk and occupying the remaining space of the egg case is the thick viscid transparent albumen. From the fact that during the artificial rearing of the embryo away from the egg case all the albumen was removed from the yolk, it is obvious that this albumen plays no part in the development or nourishment of the embryo at any stage, and purely serves the purpose of a protective cover around the embryo during its development. Its function could be regarded more or less as that of a shock absorber analogous to the function of the amniotic fluid in higher vertebrates.

The egg itself is between 30 and 35 mm. in diameter and is typical of the majority of Selachian eggs in regard to the distribution of yolk, being telolecithal. On one surface of the egg, which may be looked upon as the dorsal aspect, lies the germinal disc which is slightly lighter in color than the rest of the egg, has a diameter of 1.2 mm., and contains practically no yolk. Segmentation, as in all telolecithal eggs, is meroblastic since the cleavage planes are only restricted to the germinal disc and do not pass through the whole egg. The segmentation of the germinal disc is very similar to that of other Selachians and closely approximates that of the developing chick. The various later stages of gastrulation with the subsequent origin of the germinal layers is also typical of the class and it is not necessary to elaborate this feature. The folding off of the embryo takes place very early during development, at about 10 days after the egg is laid, the embryo then being about 2 mm. in length and attached to the yolk sac by a very well developed umbilical cord. The vitelline blood vessels at this stage are extremely prominent and spread out over the whole surface of the yolk sac. These vessels or capillaries unite into two median vessels which pass almost completely round the equator of the yolk (Plate IV, Fig. 9) to a region which lies distal from the umbilical cord. Here these vessels break up into two branches running at right angles to the equatorial vessels. These equatorial vessels pass along the sides of the umbilical cord and enter the embryo's heart.

The vitelline circulation is also typically similar to that appearing in the majority of oviparous Elasmobranchs and closely approximates that of the developing chick embryo. The umbilical cord is solid and the vitelline circulation plays a very important rôle in the transference of the nutritive food material from the yolk sac to the developing embryo. The absorption of the yolk material progressively increases with the growth of the embryo, with a concomitant decrease in the size of the yolk sac (cf., Plate IV, Figs. 7, 8 and 9). At an early stage in the development, the external gills, typical of most Selachian embryos, make their appearance

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#### PLATE IV

Stages in the external development of *H. edwardsii*.

FIGURE 6. The egg case or mermaid's purse, natural size.

FIGURE 7. A mermaid's purse opened to show a developing embryo 25 days old, natural size.

FIGURE 8. A 30-day old embryo.  $\times 1.5$ .

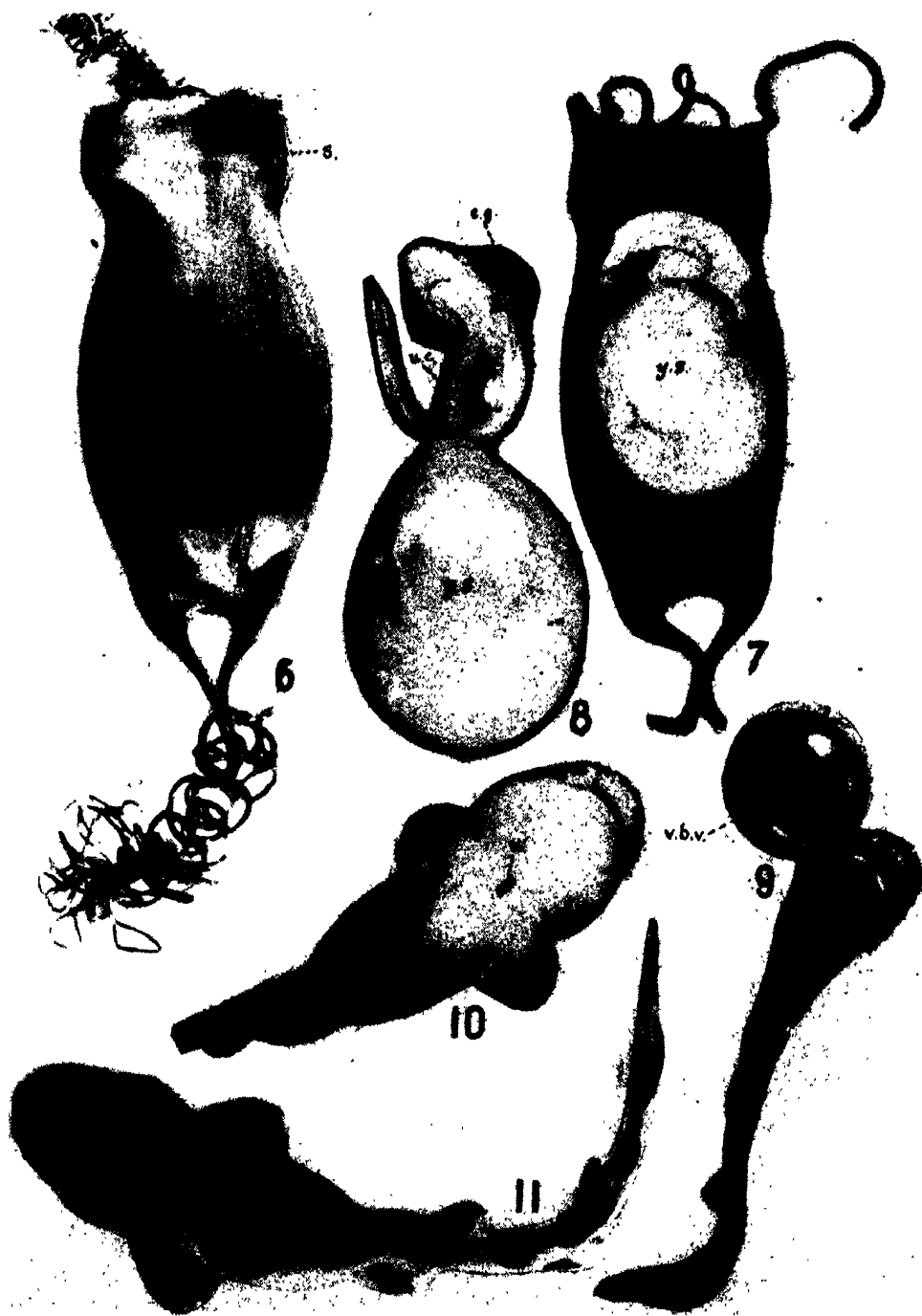
FIGURE 9. A 50-day old embryo.  $\times 1.5$ .

FIGURE 10. Ventral view of embryo at time of hatching (104 days old).  $\times 1.5$ .

FIGURE 11. Dorsal view of same.

All Photographs by Author.

PLATE IV



(Figs. 7 and 8). In origin these external gill filaments are totally unlike the external gills found in *Crossopterygii*, *Dipnoi*, and any *Amphibia*, and over their function and structure many zoologists have been puzzled.

Graham Kerr (1919, p. 157) states—"While external gills occur within three main sub-divisions of the vertebrates, namely, *Teleostomatous* fishes (*Crossopterygians*—the most archaic of existing *Teleostomes*), *Lung-fishes*, and *Amphibians* there are two main groups—*Elasmobranchs* and *Amniotes*—in which they are conspicuous by their absence. . . . As it happens, however, there is in the two groups mentioned a definite cause which seems quite competent to account for 'disappearance of external gills, namely, the development of a new organ—a yolk sac with its highly developed vitelline net work of blood vessels—which in addition to its primitive function must necessarily also function as a very efficient organ of respiratory exchange and so render any pre-existing respiratory organ no longer necessary."

In the present species these external gill filaments are richly supplied with blood vessels and in view of the fact that the embryo studied underwent its complete development in sea water outside of the mermaid's purse it is obvious that their primary function must be a purely respiratory one. The pair of slits in the mermaid's purse previously mentioned must serve for the conveyance of sea water into the interior of the purse where it probably mingles with the layer of albumen surrounding the developing embryo. Although it has been stated that these gill filaments absorb fluids which are milk-like secretions of the uterine mucosa and which serve as food for the growing embryo of all non-placental viviparous sharks and rays (Gudger, 1940) in the present species this is not one of their functions. Beard (1890, p. 310) states—"In the *Skate*-embryo the filaments are said to disappear shortly before hatching. It may be expected that their atrophy commences when the purse ruptures sufficiently to allow of the passage of sea water directly to the embryo. Then the ordinary piscine mode of respiration would be initiated, and the external gills would disappear." In the present species, however, they have already disappeared during the yolk sac stage when the embryo is 50 days old and the yolk sac has decreased to about half its original size (Plate IV, Fig. 9) and the internal gills are by this time well developed and functional.

At 104 days the embryo has absorbed all the yolk and the yolk sac has shrunk to such an extent that only a vestige of the umbilical cord remains extending through the umbilicus for a length of about 2 mm. (Fig. 10, u.). At the time of hatching the ventral abdominal surface of the embryo between the umbilicus and the cloaca is richly supplied with blood vessels which form a reticulation all over this surface (Fig. 10). The umbilicus persists for about 14 days after hatching and then completely disappears and the fish is fully formed (Fig. 11) and able to fend for itself, swimming about actively. The gestation period of the embryo which developed inside the egg case was also 104 days and the state of development at the time of hatching was the same.

#### SUMMARY

1. *Haploblepharus edwardsii* is an oviparous dogfish endemic to South African seas.
2. The male and female generative systems are dealt with and specialized features such as the albumen gland, nidamental gland, and the claspers are described.

3. Development takes place in an egg case or mermaid's purse, the gestation period lasting 104 days.
4. The development of an embryo external to the egg case is described.

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# NARCOSIS AND CELL DIVISION IN COLPODA STEINII

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## INTRODUCTION

For many years it has been known that a great number of chemical and physical agents can modify the course of mitosis and induce the development of abnormal division figures. Among the many experimental agents that have been employed to upset normal cell division are the following: basic dyestuffs (Politzer, 1924), narcotics (Politzer, 1931; Nemec, 1904; van Regemorter, 1926; Shaklevich, 1938; Ludford, 1936; Geiersbach, 1939), ether (Häcker, 1900; Schiller, 1909; Rosenfeld, 1932), alcohol (Krantz, 1938), X-radiation (Alberti and Politzer, 1923; Pfuhl and Küntz, 1939), radium (Whitman, 1933), ultraviolet radiation (Stevens, 1909), high and low temperatures (Bury, 1913; Bleier, 1930; Vintemberger, 1930; Kemp and Juul, 1931), and hypotonic solutions (Lewis, 1933). The instructive point to be gathered from a survey of such studies is that, irrespective of the agent employed, the induced morphological changes in cell division are quite similar. Thus, within certain limits of concentration or intensity of the experimental agency, disorientation of the chromosomes in the division figure, delay in polar movement and scattering of the chromosomes, "amitosis," pyknosis, dissolution of the achromatic figure, inhibition of cytokinesis, and formation of bi- and multinucleate cells are common effects.

The relative lack of specificity in results produced by such diverse experimental treatment makes the formulation of any complete explanation for these phenomena difficult. However, the suggestion of Ludford (1936) to the effect that metabolic changes may have a bearing on the production of anomalous divisional behavior becomes significant in light of recent observations indicating that special portions of the cell's metabolism are directly associated with certain physiological activity states (For details see Bodine (1934); Robbie, Boell and Bodine (1938); Deutsch and Raper (1938); Goddard and Smith (1938); Horowitz (1940); Pease (1941); Allen and Goddard (1938); van Schouwenberg (1938); Clowes and Krahle (1939); MacLeod (1941); Ormsbee (1941), and Fisher and Stern (1942)). From these studies it becomes apparent that a definite relationship exists between a portion of the over-all metabolism and cell or tissue activity. The use of narcotics as respiratory poisons and the deductions made by Fisher and his associates from data obtained by this type of treatment are of considerable significance in connection with this problem. The present study is an attempt to relate changes in cellular metabolism during division with the appearance of abnormal mitoses. The results of this study are in essential agreement with those of Fisher et al in that a respiratory parallelism can be demonstrated in *Colpoda steinii* by use of ethyl carbamate or chloral hydrate. The "activity system" of Fisher (that portion of the overall

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respiration that is most sensitive to narcotic inhibition) appears to be somehow associated with the maintenance of normal mitosis, for when it is differentially suppressed by appropriate narcotic concentrations, abnormal nuclear figures appear. These aberrant mitoses are believed comparable to those described in the literature that have been induced by many different agents. Barring such experimental intervention, the nuclear complex of *Colpoda steinii* is remarkably stable, unlike certain other members of the family Colpodidae (Burt, Kidder and Claff, 1941). Because of this stability it was selected for the study to be described.

I am indebted to Professor George W. Kidder, Arnold Biological Laboratory, Brown University, for his stimulating interest during the course of this investigation.

#### MATERIALS AND METHODS

*Colpoda steinii* was employed as experimental material throughout this investigation (for species designation see Burt, 1940). Although this ciliate cannot be readily grown in the absence of bacteria (food organisms) it offers several distinct advantages over other forms, notably, its rapid growth to high concentrations and the clarity of the mitotic changes during division (Burt, Kidder and Claff, 1941).

The ciliates were grown in sterile distilled water seeded with the coliform bacterium *Aerobacter cloacae*. One liter Erlenmeyer flasks were used as culture vessels. The procedure employed in culturing was as follows: 400 mls. of distilled water were placed in the flasks which were then plugged and autoclaved at 15 lbs. pressure for 20 minutes. Twenty-four hour Kolle flask cultures of *A. cloacae* on agar were used as food. The bacterial growth of two such Kolle flasks was harvested in 30 mls. of sterile distilled water, and 10 mls. of the resulting suspension were added aseptically by means of a pipette to each of two Erlenmeyer flasks containing the sterile distilled water. In this manner fairly uniform suspensions of food organisms were obtained. Stock cultures of *C. steinii* were carried in tubes with *Aerobacter* as food. Each flask was inoculated with one ml. of these tube cultures containing 12 hour cultures of ciliates (in the logarithmic growth phase). The Erlenmeyer culture flasks were then incubated 24–30 hours at room temperature before the ciliates were sacrificed. By adhering to this procedure an abundance of logarithmic actively dividing cells could be consistently obtained.

All culture vessels, pipettes and miscellaneous glassware employed in culturing the ciliates were autoclaved 20 minutes at 15 lbs. pressure to prevent extraneous bacterial contamination inasmuch as optimum growth of *Colpoda steinii* obtains with *Aerobacter* alone as the food organism (Kidder and Stuart, 1939). Throughout all cultural procedure rigid bacteriological technique was followed to obviate the difficulties arising from contamination of the cultures.

In the cytological studies the cells were removed from the culture flasks and centrifuged at slow speed for four minutes in 50 ml. centrifuge tubes. The supernatant was then removed by means of an aspirator and more organisms added to the tubes from the culture. Following a second centrifugation, giving adequate numbers of organisms, the ciliates were placed in 250 ml. Erlenmeyer flasks containing 50–75 ml. of freshly bacterized solution of narcotic whose effect was to be tested. Equal amounts of bacterial suspensions were added to each experimental and control solution in making up the dilution of the reagent employed. When

the desired time of treatment had elapsed (one hour) the cells were again packed, the supernatant removed, and the cells placed on coverslips by means of capillary pipettes. It was found convenient and satisfactory to employ 25 per cent acetic acid in absolute alcohol for fixation. The nuclear details were found well preserved after fixation for five minutes in this reagent. The Feulgen technique was used exclusively in making the cytological preparations. This was necessitated by the fact that differentiation of the nuclear details is difficult with haematoxylin or other stains because of the retention of the dye by the division cyst walls. Best staining results were obtained by hydrolyzing in N/1 HCl at 60° C. for 12 minutes and staining in the fuchsin sulfurous acid for six hours. The stained preparations are then washed three times for four-five minutes each in HCl-Na-Bisulfite solution, placed for ½ hour in running tap water, passed through the lower alcohols to 95 per cent and stained with fast green, dehydrated in absolute alcohol, cleared in xylol and mounted in damar.

It was customary to run these cytological experiments in four sections as follows: by centrifuging in four tubes the contents of the mass culture flasks were roughly quartered. The organisms obtained by centrifugation from three of these tubes were employed experimentally and accordingly treated with various concentrations of reagent. The cells from the fourth tube were always set up with bacterized distilled water and thus served as controls for the treated organisms. The control ciliates were always mechanically treated in a manner identical with that received by the experimental organisms. By this method aberrations in the mitotic or divisional processes could always be attributed safely to the effect of the inhibitor used.

For the respiration studies two 24–30 hour cultures set up as described were centrifuged and the cells so concentrated were resuspended in bacterized M/200 phosphate buffer at pH 7.0. One and one half mls. of this cell-buffer suspension was added to the Warburg vessels. Following equilibration (15 minutes) four readings were taken at ten minute intervals to determine the normal uninhibited respiration. The graded concentrations of inhibitor to be tested (made up in M/200 phosphate buffer at pH 7) were then dumped from the side bulbs and following a second equilibration period of ten minutes readings were again taken at ten minute intervals for one hour to determine the extent of respiratory inhibition. Temperature was controlled at 25° C.  $\pm$  0.1° C.

It was considered advisable to set up three vessels with M/200 phosphate buffer in the side bulbs to serve as controls for the experiments. In addition to these normal controls two more vessels were run with 1.5 ml. suspension of *Aerobacter* equivalent to the bacterial suspension employed in the experimental vessels. The final computed values for oxygen consumed per hour could therefore be corrected for not only an increase or decrease in normal control respiration but also for the negligible amount of oxygen consumed by the food organisms both before and after inhibition. The fact that organisms of the genus *Colpoda* encyst when the supply of food bacteria is depleted (Taylor and Strickland, 1938; Kidder and Stuart, 1939) necessitated the addition of bacteria to the experimental suspensions. Because of the presence of the bacteria, absolute values of oxygen consumed by the ciliates would be very difficult if not impossible to ascertain. However, where only relative rates of respiration are sought, as in this instance, the food organisms introduce no serious technical difficulty.

A simple technique was employed as a means of testing the degree of growth suppression in different concentrations of inhibitor. Although this method lacks the refinement of a cell counting technique, the results are believed to be roughly comparable. In these experiments four tubes were set up for every narcotic solution to be tested. The final volume of the narcotic solution following bacterization was 3 mls. In addition to this series of four tubes for every narcotic concentration tested through the range under investigation, four more tubes were set up with bacterized distilled water to serve as controls. Following inoculation with one loopful of logarithmic *Colpoda*, the growth in each of the four inhibited series and controls was read at 12, 24, and 36 hours. Control growth was arbitrarily designated as four plus, and growth in the experimental tubes designated as three plus, two plus, one plus, plus-minus, and minus depending on the degree of inhibition. The range of inhibitor concentration where growth was completely suppressed was termed the zone of complete growth inhibition. In these experiments aseptic procedure was not adhered to in either experimental or control tubes as over the period of time these determinations were made it was felt that extraneous bacteria would not significantly modify the results.

In assaying the effectiveness of the narcotic concentration tested in disrupting mitosis only cells in which the polar migration of daughter micronuclei was obviously retarded were counted as affected (compare Fig. 1-*H* with Fig. 1-*E*). As will presently be pointed out in detail, *Colpoda steinii* usually divides twice within a division cyst wall to produce four daughter ciliates. The aberrant nuclear divisions were accordingly expressed as per cent of all cells counted in mitosis between the first metaphase and the telophase of the second division. This method offered a reliable means of obtaining a quantitative estimate of the damage induced by the agents employed. It is to be emphasized, however, that the delay in polar migration of the daughter micronuclei was not the only observable defect resulting from narcosis. It merely served as a convenient method of evaluating the extent of mitotic derangement. The other changes concomitant with the polar defect will be considered elsewhere.

Potassium cyanide, potassium ferricyanide, sodium arsenite, iodoacetate, various carbamates and chloral hydrate were tested at varying concentrations for their effect on the division mechanism. Ethyl carbamate and chloral hydrate were most thoroughly studied in this respect and were also selected as inhibitors for the respiration studies.

## OBSERVATIONS

### *Normal division*

The organisms of the genus *Colpoda* normally reproduce within division cysts (for details see Kidder and Claff, 1938; Burt, Kidder and Claff, 1941). The trophic ciliates (Fig. 1-*A*) round up prior to division, dedifferentiate and secrete the cyst wall within which the ensuing fissions occur, *C. steinii* usually dividing twice to form four daughter cells. These stages are depicted in Figure 1, *A-G*. The first changes in the nuclear complex marking the onset of division are to be found in the macronucleus. This organelle loses its trophic ellipsoidal shape, becomes rounded, and the polar chromatin aggregates break up into irregular masses attaining the general configuration shown in Figure 1-*B*. Meanwhile the

micronucleus proceeds through the prophasic changes first marked by swelling then condensation of chromatin to form striae from which the chromosomes are formed. These micronuclear transformations culminate in the metaphase configuration shown in Figure 1-B with the chromosomes oriented parallel to the long axis of the spindle. At this stage the macronuclear chromatin may be considered as divided into two portions, one consisting of the irregular masses centrally disposed; the other, being peripherally located and in optical section, seems to be plastered to the macronuclear membrane giving rise to a beaded appearance. The anaphase is depicted in Figure 1-C. At the termination of metaphase, the division figure appears to break into two parts followed by the rapid movement of the daughter halves to polar positions at opposite sides of the macronucleus (Fig. 1-D to E). Upon displacement of the daughter micronuclei by  $180^\circ$  the macronucleus elongates and constricts centrally. Cytokinesis soon follows at the completion of which the daughter micronuclei immediately pass into prophase and the same series of events is repeated with the result that four daughter cells are produced as shown in Figures 1-F and 1-G. Motor and oral organelles are differentiated in the four daughters and swimming movements are taken up within the cyst wall until finally the cyst membrane is ruptured and the ciliates escape into the surrounding medium. No visible nuclear extrusion has been observed during divisional phases in this species (Burt, Kidder and Claff, 1941).

#### *Experimental modification of mitosis*

Striking differences were observed in the effectiveness of the various inhibitors employed in disrupting the divisional mechanism. Ethyl carbamate was found to be quite active in this respect, however, and because of various other advantages offered by this compound, notably its high solubility and effectiveness over a relatively wide concentration range, it was most extensively studied. Potassium cyanide, potassium ferricyanide, sodium arsenite (neutralized solution), and iodoacetate were without effect up to toxic concentrations. Related carbamates produced changes similar to those induced by ethyl carbamate and the results of treatment with chloral hydrate are believed to be entirely comparable to those caused by inhibition with the urethanes. In the following account the cytological changes induced by these substances will be considered.

**Ethyl carbamate:** Striking changes in the course of mitosis were obtained in concentrations of ethyl urethane ranging from 0.5 to 2.0 per cent. The treatment did not appear to affect a particular stage of mitosis, however, but inhibition of any divisional stage in progress at the time of exposure seemed to obtain. A similar observation was made by Ludford (1936) for this narcotic. In view of

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FIGURE 1. *Colpoda steinii*  $\times 1400$ . A, trophic organism; B-G, normal division phases; H-L, aberrant divisions produced by 1.3 per cent ethyl carbamate. B, Micronucleus in full metaphase of first division. C, Micronucleus in anaphase. D, micronucleus moving to poles. E, Micronucleus continuing polar movement; macronucleus elongating. F, Micronucleus at metaphase of second division. G, Second division completed. Nuclei of daughter cells returning to trophic condition. H, Failure of micronuclear migration; macronuclear chromatin aggregates more diffuse; reappearance of Binnenkörper. I, Similar to H; arrested polar movement of micronuclei; macronuclear chromatin diffuse; Binnenkörper material reappearing. J, Segregation of both daughter micronuclei into one cell following first division. K, Arrestment of division at micronuclear metaphase. L, Both micronuclei at one pole of elongated macronucleus.



FIGURE 1

this fact, it is not at all surprising that a great variety of aberrant divisional types were observed. Because of this almost infinite variation in the nuclear features associated with narcosis, it is exceedingly difficult to evaluate the activity of the narcotic on any other basis than its effect on polar movement of daughter micronuclei.

With ethyl urethane this polar defect is exhibited through a concentration range of 0.5 to 2.0 per cent. Thousands of cells were counted following treatment with various narcotic concentrations (for complete data on this and the following observations see Burt, 1942). In Figure 2 these data are plotted semilogarithmically. The defective polar movement increases from 0 at 0.5 per cent urethane to a maximum of around 21 per cent at 1.5 per cent urethane concentration. This maximum value is probably determined by the number of ciliates in the first or second divisions whose micronuclei are between metaphase and telophase. During these stages one aspect of inhibition of cell division is expressed by lag in poleward movement.

As pointed out, however, the effect of urethane in appropriate concentrations on dividing *C. steinii* is characterized not only by delay of polar movement, but also by changes in the state of aggregation of both macro- and micronuclear chromatin and also by abnormal configurations of the dividing nuclear complexes. Some of these changes are illustrated in Figures 1-H to 1-L.

In Figure 1-H the polar defect is very well shown. At this phase of division, judging by the elongation of the macronucleus, the daughter micronuclei should have attained polar positions in the cell as is shown in Figure 1-E, a normal division figure. On the contrary, the daughters are centrally located near the middle of the macronucleus. A somewhat similar situation obtains in Figure 1-I, although the appearance is by no means as striking. In Figure 1-L another possible orientation of the nuclear complex is shown. Here the daughter micronuclei are terminally located at the same pole. One result of such aberrant behavior on the part of the daughter micronuclei is illustrated in Figure 1-J. In this instance cytokinesis and division of the macronucleus has occurred, but the micronuclei, due to failure in assuming polar positions, have both been segregated into one daughter cell. In Figure 1-K inhibition of division has occurred at the metaphase.

In all of these cases the aggregation of the macronuclear chromatin has been changed to some extent. In general, most of the macronuclear chromatin in the treated cells exhibits a tendency to become plastered to the nuclear membrane rather than maintain a central disposition characteristic of the normal dividing nucleus. Concurrently, the aggregates become more diffuse and varying degrees of fusion are shown (Fig. 1-J).

A noteworthy feature of the urethane-treated cells is the behavior of the Binnenkörper or plasmasomal material. Normally this substance, which stains lightly with fast green in tropic ciliates, is not in evidence in preparations immediately after the onset of nuclear division. However, in narcotized cells it reappears and is found dispersed around the diffuse chromatin aggregates of the macronucleus.

The morphological behavior of the chromatin and plasmasomal material in narcotized cells is suggestive of a partial reversion to a trophic nuclear condition. It is possible that under the influence of the narcotic and consequent suppression of normal nuclear division this tendency, although normally expressed at the termination of cell division, becomes dominant. At any rate, the appearance of the inhibited cells in no way suggests an *immediate* inhibition of all nuclear activity.

It is more likely that certain changes continue and give rise to the modified division figures observed.

Indicative of the fact that any phase of cell division is susceptible to the effects of narcosis is the diversity of aberrant division figures produced as a result of treatment. A continuous series of aberrant figures representing all stages of division may be constructed.

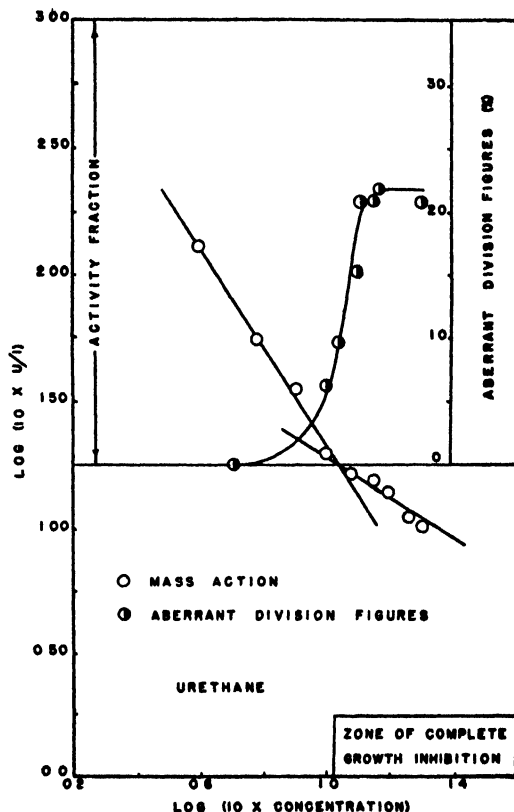


FIGURE 2. Summary of complete urethane data. Log (10  $\times$  U/I), aberrant division figures (per cent) and zone of complete growth inhibition plotted against Log (10  $\times$  concentration of urethane).

**Other carbamates:** The affects of n-propyl, phenyl, n-amyl, iso-amyl, and ethyl-n-methyl carbamate on cell division were also studied. None of these substances were tested through as wide a range of concentration in small graded increments as was ethyl carbamate. However, it was found that these closely related compounds produced divisional changes qualitatively identical with those of ethyl urethane. The ability to inhibit micronuclear migration is common to all members of this group of substances. Similarly, the associated changes in arrangement and aggregation of macronuclear chromatin and the bizarre division figures described for ethyl urethane were also induced.

**Chloral hydrate:** The activity of this compound in disturbing the normal progress of division was assayed in graded concentrations of 0.1 per cent increments between 0.05 and 0.5 per cent. The morphological changes induced were similar to those described for the urethanes, but the percentage of chloralized cells in which the polar defect appeared was never high. Moreover, the concentration range for effective induction of the polar defect is quite narrow (0.1–0.3 per cent). The maximum number of deranged mitoses was only 3.9 per cent, a small value compared with the maximum ethyl urethane value of more than 20 per cent.

In excess of 0.3 per cent chloral hydrate the delay in poleward movement and the changes in the nuclei characteristic of cells narcotized in the lower concentrations rather abruptly disappear. In these higher concentrations retrogressive changes are induced which are expressed as condensation, diffuse staining and chromatolysis of the macronucleus and pyknosis of the micronucleus. The incidence of these changes, although slight to moderate at 0.4 per cent, increases rapidly with still higher concentrations until in 0.5–0.6 per cent chloral hydrate all of the organisms, both trophic and those in division, are frankly moribund. These degenerative changes are physiologically and morphologically reversible in some cells at least after one hour exposure at 0.6 per cent. If the cells are washed free of narcotic, packed by centrifugation and resuspended in freshly bacterized distilled water, growth will ensue. It is impossible to determine, however, whether or not every cell so treated is viable.

In summary, chloral hydrate affects cell division in a manner quite similar to ethyl carbamate and related compounds. However, the effective range of concentration for the production of these changes is relatively narrow, and at no concentration are the numbers of deranged divisions high. A possible explanation for this difference in activity will be outlined.

**Effect of other inhibitors:** The following compounds in the concentrations indicated were tested for their effect on the divisional mechanism:

| Inhibitor              | Concentrations      |
|------------------------|---------------------|
| Potassium cyanide      | 0.005M to 0.000,05M |
| Potassium ferricyanide | 0.050M to 0.005M    |
| Sodium arsenite        | 0.001M to 0.000,25M |
| Monoiodoacetic acid    | 0.005M to 0.000,5M  |

These metabolic poisons throughout the concentrations designated were uniformly ineffective in the production of aberrant divisions of the type described for the carbamates and chloral hydrate. When cells were exposed to the lower concentrations of these inhibitors no change could be detected cytologically in the progress of division. However, at higher concentrations retrogressive changes characterized by extreme condensation and pyknosis of both macronuclei and micronuclei occurred. These changes were comparable to those induced at high concentrations of chloral hydrate and are considered as definite signs of cell damage as opposed to the milder type of change occurring with moderate narcotic concentrations.

### *Growth inhibition*

As a correlative procedure in connection with the cytological and metabolic studies, the effect of ethyl urethane and chloral hydrate on suppression of growth in cultures of *C. steinii* was tested.

In Figure 3 are tabulated the results from one typical determination with ethyl urethane. Fairly close correspondence in the degree of inhibition among the four experimental series was obtained. It is apparent from these results that growth is only slightly affected by 0.5 per cent urethane (3 plus to 4 plus growth). However, with increasing concentrations of narcotic, suppression of growth becomes more pronounced until at 1.0 per cent urethane no cell division at all can be detected. Similarly, Ormsbee (1941) found that 1.0 per cent urethane would completely inhibit the growth of populations of the ciliate *Tetrahymena geleii*.

| CHLORAL HYD.<br>SER-PERCENT<br>IES CONC. | EXP. 1 |      |      | EXP. 2 |      |      | EXP. 3 |      |      | EXP. 4 |      |      |
|--|--------|------|------|--------|------|------|--------|------|------|--------|------|------|
|  | HOURS  |      |      | HOURS  |      |      | HOURS  |      |      | HOURS  |      |      |
|  | 12     | 24   | 36   | 12     | 24   | 36   | 12     | 24   | 36   | 12     | 24   | 36   |
| A .025                                   | ++++   | ++++ | ++++ | ++++   | ++++ | ++++ | ++++   | ++++ | ++++ | ++++   | ++++ | ++++ |
| B .05                                    | ++++   | +++  | +++  | +++    | +++  | +++  | +++    | +++  | +++  | +++    | ++   | +++  |
| C .1                                     | +      | ±    | ±    | ±      | +    | ±    | ±      | ±    | ±    | ±      | ±    | ±    |
| D .2                                     | -      | -    | -    | -      | -    | -    | -      | -    | -    | -      | -    | -    |
| E .3                                     | -      | -    | -    | -      | -    | -    | -      | -    | -    | -      | -    | -    |
| F .4                                     | -      | -    | -    | -      | -    | -    | -      | -    | -    | -      | -    | -    |
| G CONTROL                                | ++++   | ++++ | ++++ | ++++   | ++++ | ++++ | ++++   | ++++ | ++++ | ++++   | ++++ | ++++ |

FIGURE 3. Inhibition of growth in cultures of *Colpoda steinii* with various concentrations of ethyl carbamate. Four plus growth represents growth in untreated controls.

| URETHANE<br>SER-PERCENT<br>IES CONC. | EXP. 1 |      |      | EXP. 2 |      |      | EXP. 3 |      |      | EXP. 4 |      |      |
|--------------------------------------|--------|------|------|--------|------|------|--------|------|------|--------|------|------|
|                                      | HOURS  |      |      | HOURS  |      |      | HOURS  |      |      | HOURS  |      |      |
|                                      | 12     | 24   | 36   | 12     | 24   | 36   | 12     | 24   | 36   | 12     | 24   | 36   |
| A 0.5                                | +++    | ++++ | ++++ | ++++   | ++++ | ++++ | +++    | ++++ | ++++ | ++++   | ++++ | ++++ |
| B 0.8                                | +      | +    | +    | +      | ++   | ++   | +      | ++   | ++   | +      | +++  | +++  |
| C 0.9                                | ±      | +    | +    | ±      | ±    | +    | ±      | ±    | +    | +      | +    | +    |
| D 1.0                                | ±      | ±    | ±    | ±      | ±    | ±    | ±      | ±    | ±    | ±      | ±    | ±    |
| E 1.1                                | -      | -    | -    | -      | -    | -    | -      | -    | -    | -      | -    | -    |
| F 1.2                                | -      | -    | -    | -      | -    | -    | -      | -    | -    | -      | -    | -    |
| G CONTROL                            | ++++   | ++++ | ++++ | ++++   | ++++ | ++++ | ++++   | ++++ | ++++ | ++++   | ++++ | ++++ |

FIGURE 4. Inhibition of growth in cultures of *Colpoda steinii* with various concentrations of chloral hydrate. Four plus represents growth in untreated controls.

In Figure 4 are represented the results of an analogous determination made with chloral hydrate. Here complete inhibition of growth takes place in 0.2 per cent of the narcotic.

Comparison of the two sets of experimental results is instructive in indicating the differences in effective concentration ranges of the two inhibitors. In the case of ethyl carbamate an increment of 0.6 per cent in concentration must be made from the point where its effect is first noted until complete inhibition occurs (from 0.5 to 1.1 per cent). With chloral hydrate this increment is only 0.15 per cent or one fourth that of urethane. This is correlated with the brief range of effectiveness of chloral hydrate in the induction of aberrant division figures.

### *Respiratory metabolism*

As pointed out in a preceding section, overall oxygen consumption in a cell or tissue does not necessarily have uniform significance as regards a specific function. Perhaps the most convincing evidence for this is the interpretation of narcotic inhibition data made by Fisher and his colleagues (for review see Fisher, 1942).

Equations expressing the relationship between enzyme and inhibitor may be derived from the Law of Mass Action (Warburg, 1927; Warburg and Negelein, 1928; Fisher and Öhnell, 1940). As required by the Mass Action formulation, when the logarithm of the concentration of inhibitor (narcotic) is plotted against the logarithm of  $\frac{\text{Uninhibited respiration}}{\text{Inhibited respiration}}$  a straight line should result. Non-linearity of mass action curves so constructed is believed to indicate the presence of at least two parallel respiratory systems in *Arbacia* eggs and in yeast (Fisher, 1941a, b; Fisher and Henry, 1940; Fisher and Stern, 1942).

The respiration of *Colpoda steinii* was tested in graded concentrations of urethane and chloral hydrate with the object of obtaining evidence for such respiratory discontinuity in this organism. The results of respiratory inhibition as measured by the Warburg technique between concentrations of 0.4 and 2.0 per cent urethane are plotted in Figure 2. At a concentration of urethane between 1.0 and 1.2 per cent a break in the mass action curve occurs at which point respiration is inhibited by 35 per cent. This is taken as evidence for the existence of two separate respiratory mechanisms that differ in sensitivity to narcotic inhibitor.

In Figure 2 is also shown the relationship between this respiratory data, growth inhibition, and aberrant nuclear behavior at various levels of narcotic inhibition. The increase in pathological cell divisions is related to the degree of suppression of the upper limb of the mass action plot representing the inhibition of the more highly narcotic-sensitive fraction of metabolism. It is also interesting to note the correspondence between the concentration of urethane giving complete growth inhibition (1.1 per cent) and the concentration at which the break in the mass action curve occurs (approximately 1.1 per cent).

A discrepancy exists in these relations, however, for the maximum number of aberrant nuclear divisions is not obtained until a concentration of 1.4 to 1.5 per cent urethane is reached. This may be due to experimental error in part, but the theoretically more attractive possibility is that an overlapping in the inhibition of the two respiratory systems occurs. In accordance with this view the resting system would begin to be inhibited before the activity system was completely suppressed. This point will be dealt with more fully in a following section. Apparently, however, following complete inhibition of the more narcotic sensitive fraction (activity system of Fisher) not only is growth of the culture suppressed, but the incidence of abnormal divisional figures attains a maximum.

In Figure 5 is summarized the effect on respiration, growth and the derangement of cell division brought about by graded concentrations of chloral hydrate between 0.0125 and 0.6 per cent. These results resemble those obtained with ethyl urethane. Non-linearity of the mass action curve is characteristic. Also a fair correspondence exists between the concentration giving complete growth inhibition (0.2 per cent) and the concentration at which discontinuity of the respiratory inhibition becomes apparent (0.1 per cent). Unlike urethane, however, the per-

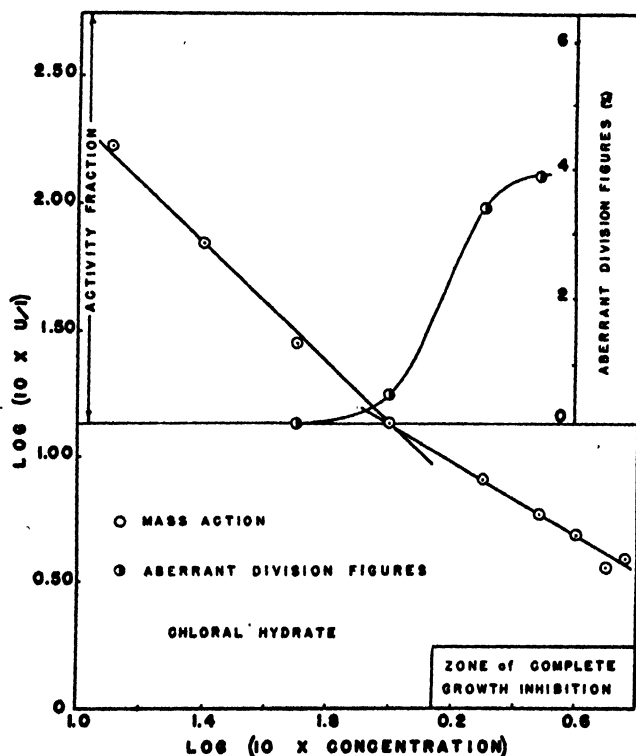


FIGURE 5. Summary of complete chloral hydrate data. Log (10  $\times$  U/I), aberrant division figures (per cent) and zone of complete growth inhibition plotted against Log (10  $\times$  concentration of chloral hydrate).

centage of aberrant nuclear divisions induced by chloral hydrate is never high, the maximum being in the vicinity of 4 per cent. This maximum is not obtained until the cells are exposed to 0.3 per cent chloral hydrate, or 0.2 per cent in excess of the concentration at which discontinuity in the respiratory data is expressed. Again, a possible explanation for this may be an overlapping in the inhibition of the activity and resting systems whereupon after a certain concentration is exceeded, both are inhibited simultaneously. That the more sensitive fraction indicated by chloral hydrate represents about 45 per cent of the total respiration (taking the break in the curve as the end point) in contrast to 35 per cent with urethane makes this possibility plausible. The higher value of respiratory inhibition, down to the

point where discontinuity is displayed in the mass action curve therefore represents the activity system plus the added respiratory decrease caused by the suppression of part of the less sensitive fraction.

### DISCUSSION

It is believed that under the experimental conditions adhered to in this study the data indicate the presence of at least two fractions of metabolism in *Colpoda steinii*. These differ in their sensitivity to inhibition by either chloral hydrate or urethane. One portion of this parallel respiratory mechanism, the activity fraction, appears to be associated with the maintenance of normal cell division. In general, this is in agreement with the observations of Fisher and his colleagues (1940-1942). This conception is based on the fact that discontinuity in the relation between concentration of narcotic and its effect in depressing respiration of these ciliates is expressed by mass action treatment of the data. The factors that might invalidate such an interpretation have been pointed out by Fisher and Stern (1942), but it is believed that these factors were not operative in this investigation.

Complete and quantitative separation of activity and resting fractions of the total metabolism by differential narcotic inhibition is considered here as extremely unlikely. Four findings in this report support the view that parallel inhibition of the two systems occurs in an intermediate range of narcotic concentrations:

1. The increase to a maximum in the incidence of nuclear aberrations after mass action discontinuity has been expressed. This is considered most significant and was found with both urethane and chloral hydrate (See Figs. 2 and 5).
2. The difference of 10 per cent between the activity respiration indicated by urethane (35 per cent) and that of chloral hydrate (45 per cent).
3. The delay in obtaining complete growth suppression with chloral hydrate after discontinuity in the mass action curve has been expressed.
4. The brief effective concentration range of chloral hydrate in inducing abnormal division figures and the low maximum for the polar defect obtained with this reagent.

Nuclear divisional activity persists at narcotic concentrations in excess of those at the break in the mass action curves. The maximum number of nuclear abnormalities with urethane does not occur until a concentration of 1.5 per cent of the narcotic is reached, and 0.3 per cent chloral hydrate is necessary to attain the corresponding maximum. Hence, the relation between the activity fraction, as determined by the respiratory inhibition curves, and divisional activity is not precise. It is believed that this failure of complete correspondence between the production of aberrant mitoses and discontinuity of the respiratory data is due to parallel or simultaneous inhibition of both activity and resting systems in an intermediate range of narcotic concentrations. In effect, this means that continued nuclear activity is possible even after the break in the mass action curves occurs because the activity system is not completely suppressed at this point. Mitosis therefore continues in an increasingly abnormal fashion until the activity metabolism is finally completely eliminated.

The conception of overlapping or parallel inhibition of both respiratory mechanisms also implies that the slopes of the two lines describing the reactions cannot

be accurately descriptive of the relation between the narcotics on the separate systems even though the existence of at least two respiratory mechanisms is apparent. Furthermore, the value of 35 per cent representing the activity respiration, as determined by urethane inhibition, is not an absolute value for the percentage of the total energy involved in cell multiplication. However, this figure corresponds fairly well with the 28 per cent found for *Tetrahymena geleii* by Ormsbee (1941) and the 30-40 per cent of the overall respiration associated with the maintenance of cell division in sea urchin eggs reported by Krah1 and Clowes (1939).

The value of 45 per cent for activity respiration, determined by chloral hydrate, can be accounted for by overlapping in the inhibition of the two respiratory mechanisms. The additional 10 per cent activity respiration indicated by this drug would thus represent part of the resting respiratory mechanism that was involved at the narcotic concentration where the break in the mass action curve occurred. Failure to completely suppress growth at a concentration of 0.1 per cent chloral hydrate (at break in logarithmic plot) likewise is indicative of this inhibition overlap. With the increase in narcotic to 0.2 per cent cell multiplication ceases because of the more complete elimination of the activity system.

When the progress of mitosis is suppressed by appropriate narcotic concentrations nuclear changes described earlier in this report as reorganizational are induced. Higher concentrations of narcotic inhibit these trophic changes (as 3 per cent urethane or 0.5 per cent chloral hydrate). At these concentrations the nuclei become condensed and pyknotic. The morphology described as resulting from varying degrees of suppression of the activity system obtained at lower narcotic concentrations are not seen at these higher drug levels. Thus in the production of nuclear abnormalities of the type described, two mechanisms appear to operate:

1. The inhibition of the activity system with associated suppression of the progress of mitosis.
2. Reorganization of the nuclei toward the trophic condition at that stage in division where inhibition has occurred.

The trophic changes are considered here to be dependent upon the maintenance of the resting metabolism of the cell (narcotic resistant system). As more of this less sensitive respiratory system is inhibited with increasing narcotic dosage, a point is finally reached when reorganization toward the trophic morphology cannot occur. Concomitantly pyknosis sets in and the nuclear picture is completely altered. Where the resting fraction is inhibited simultaneously with the activity fraction a restriction on the effective production of aberrant divisional figures results. The brief range of effectiveness and low maximum for nuclear abnormalities obtained with chloral hydrate can be accounted for on this basis.

The conception of overlapping with respect to narcotic inhibition postulated in the foregoing account is supported by the observation of Fisher (1941a) that benzoate inhibits the resting system in sea urchin eggs before inhibiting the activity system. Here it is necessary to inhibit 50 per cent of the respiration before inhibition of cleavage occurs. The condition described for *C. steinii* in its behavior with narcotics appears to represent an intermediate situation between the theoretical, where precise quantitative separation of the two systems would occur, and the juxtaposition of the two systems with respect to sensitivity to benzoate described for sea urchin eggs.

It is to be emphasized that the narcotic concentration required to inhibit cell multiplication in cultures corresponds closely with that at the point in the mass action curves where discontinuity is expressed, i.e., when the activity system, as determined by this method, is inhibited growth of the culture ceases. To suppress nuclear activity, however, more complete elimination of the activity system appears to be necessary, hence the delay in attaining the maximum number of abnormal division figures.

The cytological effects of narcotics on dividing *Colpoda steinii* are considered here as unique, but are comparable to the results obtained with these agents on many other cell types. Furthermore, it is apparent that in addition to narcotics many chemical and physical agents induce quite similar changes in the course of cell division. In general, the interpretation placed on this non-specificity of effect is that regardless of the agent employed there is somehow brought about a modification of the intracellular colloid state. This common result of experimental treatment would account for the similarities of morphological variation (see Ludford, 1936; von Lehotzky, 1938; Kemp and Juul, 1931; M. R. Lewis, 1933, 1934; Rosenfeld, 1932). This point of view, in light of the paucity of information available that is pertinent to the actual mechanics of these defects, appears to be well taken. However, the complexity of the protoplasmic organization in its reactions to various treatments must not be overlooked.

In the present study it has been shown that with gradual suppression of that portion of the cells' energy yielding reactions designated as activity metabolism, cell multiplication is suppressed and abnormal division figures are induced. It is not surprising that the divisional mechanisms drawing on these energy sources should reflect such reductions in energy production by abnormal behavior. However, it is difficult to visualize the manner in which this metabolic inhibition becomes morphologically expressed.

Possibly by interfering with cellular oxidation (narcosis) physical changes in the protoplasm result (von Lehotzky, 1938); this would relate the abnormal division figures obtained by narcotics to those induced by temperature changes, ether, carbon dioxide, hypo- and hypertonic solutions. All of these agencies have been demonstrated to affect the viscosity of protoplasm (for review, see Chamber's discussion in Cowdry's *General Cytology*). Conceivably, such experimental interference might exert its effect by modifying normal cyclical viscosity changes occurring during cell division (Heilbrunn, 1917; 1920; 1921).

No doubt such physical changes are significant for the normal progress of cell division and bear consideration in dealing with experimentally induced changes in mitosis. That such purely physical aspects fail to describe the entire sequence of events encountered in the complex dynamics of cell division is obvious. It may be, however, that they are the links between the biochemical phases involving cellular oxidations and the well known cytological manifestations of activity. Integrating factors for such an association are at present lacking. With regard to the present study, it can only be said that a certain portion of the overall metabolism is probably associated with these mechanisms. To postulate more than this would be the sheerest speculation.

Briefly, the negative results obtained in the preliminary work with cyanide, ferricyanide, arsenite and iodoacetate may be considered. The reactions of any of these inhibitors in the intact cell, with the possible exception of cyanide, are

complex. At the present time data concerning their effect on the metabolism of *Colpoda steinii* are lacking. Apparently, however, with the limited information concerning the morphological expression of treatment by these agents, their effects differ considerably from those of the narcotics tested. To take one example, cyanide does not seem to visibly alter the division mechanism until the occurrence of retrogressive changes resembling those induced by the higher doses of narcotics. Tentatively it may be suggested that cyanide, by virtue of its reaction at the oxygen end of the cellular oxidation chain where a large percentage of respiration is mediated, simultaneously inhibits both activity and resting systems. When a certain percentage of the resting metabolism has been suppressed, the retrogressive alterations are induced.

#### SUMMARY

1. Ethyl carbamate has been demonstrated to be effective in inducing aberrant nuclear behavior during division of the ciliate *Colpoda steinii*. This activity is shared by other carbamates and chloral hydrate. These effects are described in detail.
2. A quantitative means of assaying cytologically the activity of these substances is described. On the basis of defective polar movement of daughter micronuclei ethyl carbamate was found to be more active than chloral hydrate. A possible explanation for this observation is postulated.
3. The results of preliminary work involving treatment of dividing cells with potassium cyanide, potassium ferricyanide, arsenite, and iodoacetate are reported. These agents apparently do not share the activity evidenced by the carbamates or chloral hydrate.
4. By means of the Warburg technique the effect of urethane and chloral hydrate on respiration of *C. steinii* was studied.
5. Discontinuity in the quantitative action of these agents on respiration is interpreted to indicate the existence of at least two fractions of metabolism. One of these, the activity system, is believed to be associated with the process of cell division.
6. Complete separation of these two systems is not considered probable, however, and caution is urged in the interpretation of discontinuity in Mass Action treatment of this and similar data.
7. The significance of the association of the appearance of aberrant division figures, growth inhibition and discontinuity in the relationship of narcotic concentration to respiratory inhibition is pointed out. Discrepancies in these relationships are discussed.
8. The bearing of metabolic inhibition on the general problem of the pathology of mitosis is discussed.

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A NOTE ON THE ABSORPTION SPECTRA OF THE BLOOD OF  
EUDISTYLIA GIGANTEA AND OF THE PIGMENT IN THE  
RED CORPUSCLES OF CUCUMARIA MINIATA  
AND MOLPADIA INTERMEDIA

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In 1868 Lankester noted the presence of a red pigment (erythrocrucorin) in the plasma of certain annelids, in Chironomus larvae and in Planorbis. He also reported a green pigment (chlorocrucorin) in the plasma of Siphonostoma and Sabella. Subsequent studies have revealed the widespread occurrence of erythrocrucorin<sup>2</sup> (invertebrate hemoglobin) in many worms, in echinoderms (holothuroids), in some molluscs and in a few arthropods (Redfield, 1933; Kobayashi, 1936).<sup>3</sup> Chlorocrucorin appears to be more limited in its distribution having been found thus far only in certain polychaete worms (Fox, 1925).

The absorption curves of both these heme pigments have been studied in some detail by Fox (1925), Redfield and Florkin (1931) and Kobayashi (1932, 1935, 1936). From a review of this literature it appears that the  $\alpha$ -band of oxyerythrocrucorin falls, in most cases, well within the 577–579  $m\mu$  region, although figures as low as 574.5  $m\mu$  have been reported for some of the worms (Barcroft and Barcroft, 1924; Kobayashi, 1936). The usual position of the  $\alpha$ -band agrees well with the position of the comparable band in vertebrate blood. The  $\beta$ -band of oxyerythrocrucorin occurs, in most cases, between 540–542  $m\mu$  although, here again, there are exceptions as in the case of the holothuroid, *Caudina chilensis*, where this band is found at 544.2  $m\mu$  (Kobayashi, 1932) and in the case of the earthworms, *Pheretima communissima* and *Pheretima hilgendorfi*, where the band occurs at 538  $m\mu$  and 539  $m\mu$  respectively (Kobayashi, 1936). The usual position of the  $\beta$ -band of oxyerythrocrucorin agrees generally with the position of the comparable band in vertebrate blood. Reduced erythrocrucorin, in many cases, possesses a single band with maximum at 556  $m\mu$ , which is also the case for vertebrate hemoglobin, but in *Caudina chilensis* this maximum has been reported to occur at 560  $m\mu$  (Kobayashi, 1932) and in *Cucumaria frauenfeldi* at 558  $m\mu$  (Hogben and Van Der Lingen, 1938). The situation appears to be distinctly different for some of the worms where the reduced pigment possesses a double peak; one band being at 566–571  $m\mu$ , the other at 549–551  $m\mu$  (Kobayashi, 1936; Vlès, cited by Kobayashi, 1936).

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<sup>2</sup> Many authors, since the original work of Lankester, have used the term hemoglobin for the pigment in invertebrates, but the present writer is following the suggestion of Svedberg and Eriksson (1933) that, since the protein portion of invertebrate hemoglobin is characteristically different from that of vertebrate hemoglobin, the separate name, erythrocrucorin, is justified.

<sup>3</sup> A report has been published by Sato and Tamiya (1937) indicating bands of hemoglobin in *Paramecium caudatum*, but the writer has seen only an abstract of this report.

Compared to oxyerythrocruorin, the bands of oxychlorocruorin are shifted toward the red end of the spectrum. In five species of worms, Fox (1925) obtained a spectrum with the  $\alpha$ -band in the 602.5–605.9  $m\mu$  region, while the  $\beta$ -band (in *Spirographis*) is at 561  $m\mu$  and a third faint band (also in *Spirographis*) is located at about 517  $m\mu$ . Using crystalline chlorocruorin, Roche and Fox (1933) have been able to confirm these results.

Recently the author had occasion to examine spectroscopically the green pigment dissolved in the plasma of the tube worm, *Eudistylia gigantea*, and the red pigment of the corpuscles found in the perivisceral fluid of the holothuroids, *Cucumaria miniata* and *Molpadia intermedia*. The results which were obtained agree in many details with previous data, but in some respects the results are so strikingly different that a record of them would be of value, even though the war has prevented completion of the investigation.

The author wishes to express his appreciation to Professor Thomas G. Thompson for making available the facilities at the laboratories in Friday Harbor and the Oceanographic Laboratory in Seattle. To Professor Trevor Kincaid appreciation is expressed for his aid in identifying the animals used in this work.

#### MATERIALS AND METHODS

The large tube worm *Eudistylia gigantea* (family Sabellidae) is found at relatively low tidal levels in large colonies attached to rocks or pilings in certain areas of Puget Sound. The pigment, which is dissolved in the plasma, appears red in the concentration occurring in the dorsal blood vessel, but upon removal and dilution it is seen to be green. About 0.1–0.2 ml. of blood was obtained by means of a glass capillary tube inserted into the dorsal blood vessel. After dilution to about 50–100  $\mu$ l. with either cold distilled water or with cold sea water, the solution was filtered and the filtrate, containing the pigment, was placed in a 10 cm. long specimen tube. The spectrum was then obtained by visual matching, using a Bausch and Lomb spectrophotometer. The holothuroids which were used include the sea cucumber, *Cucumaria miniata* (order Dendrochirotida), which is found abundantly at Friday Harbor in between rocks at low tide level, and the apodous sea cucumber, *Molpadia intermedia* (order Molpadiida), which was obtained from the muddy bottom of East Sound at a depth of 12–15 fathoms. The red pigment is located in numerous corpuscles suspended in the perivisceral fluid of both these sea cucumbers. These corpuscles in *Molpadia*, when examined microscopically, are seen to be pale-yellow cells which possess a variety of elongated, multi-lobed shapes. A few are oval or spherical in form and all of them have a single, small, dark and spherical nucleus located at a variety of points in the cell but rarely at the geometric center. When a drop of distilled water is mixed with a drop of the perivisceral fluid the cells assume a perfectly spherical form and the nucleus is seen to occupy an eccentric position. The corpuscles of *Cucumaria* are also pale-yellow, nucleated cells with a variety of shapes; some are ovoid, some spherical and others are quite irregular with one or several processes. The elongated lobed forms of *Molpadia* are seldom seen in *Cucumaria miniata*. Previous accounts of the holothurian red cell by Dawson (1933) and Ohuye (1936) have already mentioned some of these structural features. By means of a small puncture in the body wall, the red perivisceral fluid was collected and the cells cen-

trifuged out. After decanting the supernatant fluid, the cells were washed in cold sea water and again centrifuged out. This process was repeated three or four times after which the cells were hemolyzed in cold distilled water. The solution of pigment after filtration and dilution to about one hundred ml. with cold distilled water was examined spectroscopically.

### Results and discussion

The five curves (Fig. 1) obtained from five different sea cucumbers (*Cucumaria miniata*) give an indication of the reproducibility of the spectral curve and show that the location of a point of maximum or minimum can be checked within  $2\text{ m}\mu$ . These curves, which were obtained from well-aerated solutions of the pigment, show two maxima; one  $580\text{--}581\text{ m}\mu$ , the other at  $544\text{--}545\text{ m}\mu$ . The point

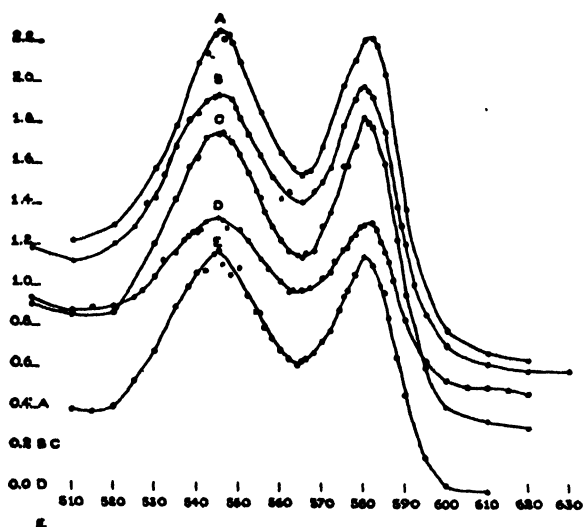


FIGURE 1. The absorption spectral curves of the pigment from five different sea cucumbers (*Cucumaria miniata*). The density ( $\log I_0/I$ ) is plotted as ordinates against the wave length, in  $\text{m}\mu$ . The individual graphs are plotted on a multiple ordinate scale to avoid crowding. The zero ordinate for each curve is indicated by the corresponding letter. Each plotted point represents the mean of 3-5 spectrometric readings.

of minimum absorption between these two maxima is at  $564\text{--}565\text{ m}\mu$ . A comparison of the spectral curves of the pigments, in the oxidized state, from all three species is shown in Figure 2. It is evident that all three pigments show two maxima, but the maxima for Eudistylia and for Molpadia are shifted toward the red end of the spectrum when compared with the *Cucumaria* data, the shift being greater in the case of Molpadia. The position of the maximum and minimum points for all the curves that were obtained from all three organisms are listed in Table I.

Reduction of the pigment by means of sodium hyposulphite results in a radical, though reversible, change in the spectral curve for all three pigments (Fig. 3). In the case of *Cucumaria* the original bands disappear and two new bands, one at  $562\text{--}563\text{ m}\mu$ , the other at  $530\text{--}532\text{ m}\mu$ , make their appearance. The point of mini-

mum between these two maxima is located at  $545\text{ m}\mu$  (Table I). In *Eudistylia*, reduction results in the disappearance of the two original bands and in the appearance of a band with a peak at  $577\text{--}580\text{ m}\mu$ . There is also an indication of a secondary band at about  $540\text{ m}\mu$  (Fig. 3) but the data on this point are too meagre to merit any degree of confidence. Reduction of the pigment from *Molpadia*, again with hyposulphite, leads to the replacement of the two original bands by a single band at  $588\text{ m}\mu$ . It is possible that, here too, more data would reveal the existence of a secondary band since an indication of this is visible in the curve for *Molpadia*

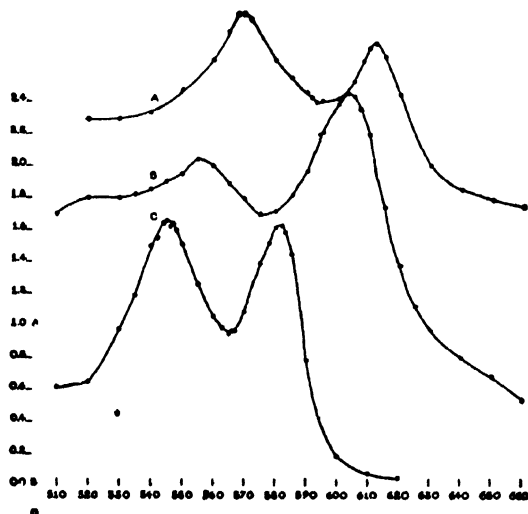


FIGURE 2. The absorption curves of the oxidized pigment from *Molpadia intermedia* (A), *Eudistylia gigantea* (B), and *Cucumaria miniata* (C). The curves are plotted on a multiple ordinate scale. Other details as Figure 1.

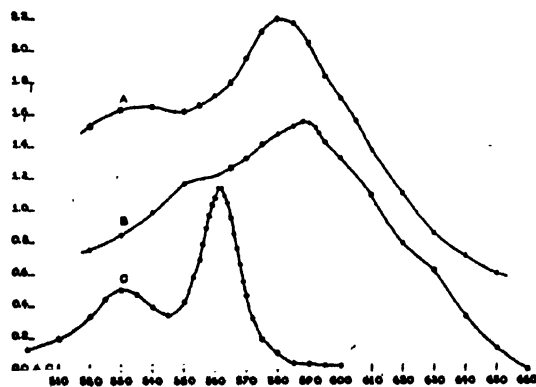


FIGURE 3. The absorption curves of the pigment after reduction with sodium hyposulphite from *Eudistylia gigantea* (A), *Molpadia intermedia* (B), and *Cucumaria miniata* (C). The curves are plotted on a multiple ordinate scale. All other details as in Figure 1.

TABLE I

| Species *                                   | Oxidized state |         |           | Reduced state |           |
|---|----------------|---------|-----------|---------------|-----------|
|   | $\alpha$       | $\beta$ | Minimum * | Main          | Secondary |
| <i>Caudina chilensis</i>                    | 579.5          | 544.2   | 564.5     | 560           |           |
| <i>Molpadia roretzii</i>                    | 577            | 541.5   | 562       | 557           |           |
| <i>Cucumaria frauenfeldi</i>                | 579            | 543     |           | 558           |           |
| <i>Spirographis spallanxi</i><br>(crystals) | 602.5          | 562.5   |           |               |           |
| <i>Eudistylia gigantea</i> 1                | 605            | 555     | 576       | 580           | 540       |
| 2   | 604            | 555     | 576       | 577           |           |
| 3   | 603            | 558     | 577       |               |           |
| 4   | 602            | 554     | 575       |               |           |
| <i>Cucumaria miniata</i> 1                  | 581            | 545     | 564       | 562           |           |
| 2   | 580            | 545     | 564       | 561.5         | 530       |
| 3   | 580            | 544     | 564       | 562           | 531       |
| 4   | 580            | 544     | 565       | 562           | 531       |
| 5   | 580            | 547     | 565       | 562           | 531       |
| 6   | 582            | 545     | 565       | 563           | 532       |
| 7   |                |         |           | 563           | 535       |
| <i>Molpadia intermedia</i> 1                | 615            | 570     | 592       | 588           |           |
| 2   | 612            | 570     | 592       | 588           |           |
| 3   | 611            | 568     | 595       |               |           |

\* The position of the minimum between the  $\alpha$ - and  $\beta$ -bands.

The data from *Caudina* and *Molpadia roretzii* were taken from Kobayashi (1932).

The data for *Cucumaria frauenfeldi* were taken from Hogben and Van Der Lingen (1928).

The data for *Spirographis* were taken from Roche and Fox (1933).

(Fig. 3). It is clear, then, that reduction of the pigment from all three organisms leads to a hypsochromic shift, but the shift is not one of equal magnitude for all three organisms, since the span from the  $\alpha$ -band to the principal band of the reduced pigment is about 18  $m\mu$  for *Cucumaria*, 25  $m\mu$  for *Molpadia* and 26  $m\mu$  for *Eudistylia*.

It seems very likely that the pigment in the red cells of *Cucumaria miniata* is erythrocrucorin. The positions of the  $\alpha$ - and  $\beta$ -bands, as well as the point of minimum, agree reasonably well with the previous data obtained with other holothurians (Table I). The reduced pigment from *Cucumaria* shows two bands; the main band at 562–563  $m\mu$  agrees approximately with the 557–560  $m\mu$  band previously reported for holothurians (Table I), but the secondary band at 530–532  $m\mu$  is previously unreported, although from the plasma pigment of certain worms (Kobayashi, 1936) a spectral curve has been obtained which has a 2-banded structure with the secondary band in the 549–551  $m\mu$  region.

It also seems likely that the green pigment dissolved in the plasma of *Eudistylia gigantea* is chlorocrucorin. The  $\alpha$ -band at 602–605  $m\mu$  (Table I) agrees completely with the data given by Fox (1925) and by Roche and Fox (1933), although the present data shows the  $\beta$ -band to be shifted toward the violet end of the spectrum by about 6–7  $m\mu$  as compared with the position of the  $\beta$ -band given by the above-

mentioned workers. The principal band of the reduced chlorocruorin from *Eudistylia* has a peak at 577–580  $m\mu$  whereas the data of Fox (1925) for *Spirographis* place it at about 574  $m\mu$ .

The results obtained from *Molpadia intermedia* are surprising. At the outset there was no reason to suspect that an absorption curve agreeing approximately with that obtained by Kobayashi (1932) from *Molpadia roretzii*, and indicating an erythrocrurorin, should not be obtained. Instead, as Figures 2 and 3 indicate, the bands for both the oxidized and reduced pigment are shifted toward the red end to a degree even greater than in the case of chlorocruorin. The  $\alpha$ -band of *Molpadia intermedia* occurs 36  $m\mu$  further toward the red than the corresponding band for *Molpadia roretzii*, while the band of the reduced pigment of *Molpadia intermedia* is 31  $m\mu$  further toward the red than in *Molpadia roretzii*. It is also significant to note that the span between the  $\alpha$ - and  $\beta$ -bands in other sea cucumbers (Table I) is 35–36  $m\mu$  whereas the corresponding span in *Molpadia intermedia* is about 43  $m\mu$ . These differences appear to be too great to be accountable to errors in measurement or to the usual species differences that are known to occur. The spectrum of the pigment from *Molpadia intermedia* does not agree with the spectrum of hemerythrin, the red pigment found in certain Gephyrean worms as well as in the polychaete, *Magelona* (Marrian, 1927). It must be concluded that either this represents the true absorption spectrum of a heme pigment (if it is a heme pigment) characteristically different from either erythrocrurorin or chlorocruorin, or that unrecognized conditions cause a shift of the bands from the typical positions of erythrocrurorin. The onset of war resulted in the sudden interruption of the investigation at this point so that this final question must remain unanswered till a later date. A thorough examination of the crystallized pigment by chemical as well as by spectroscopic means should be made before the existence of a new pigment can be accepted.

#### SUMMARY

A spectrometric examination of the green pigment dissolved in the plasma of the tube worm, *Eudistylia gigantea*, and of the red pigment in the corpuscles of the sea cucumbers, *Cucumaria miniata* and *Molpadia intermedia*, has led to the following conclusions.

1. The green pigment of *Eudistylia* appears to be chlorocruorin. In the oxidized state it possesses an  $\alpha$ -band at 602–605  $m\mu$  and a  $\beta$ -band at 554–555  $m\mu$ . In the reduced condition a main band occurs at 577–580  $m\mu$  with a second band suggested at 540  $m\mu$ .
2. The red pigment of *Cucumaria* appears to be erythrocrurorin. In the oxidized state an  $\alpha$ -band at 580–581  $m\mu$  and a  $\beta$ -band at 544–545  $m\mu$  are seen. When the pigment is reduced a band appears at 562–563  $m\mu$  as well as one at 530–532  $m\mu$ .
3. The red pigment of *Molpadia*, when oxidized, possesses a band at 611–615  $m\mu$  and another at 568–570  $m\mu$ . In the reduced condition a band at 588  $m\mu$  is evident. This spectrum does not agree with the spectrum of either chlorocruorin or of erythrocrurorin. The new spectrum may indicate the existence of another pigment with the ability to combine reversibly with oxygen.

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# GUSTATORY REJECTION THRESHOLDS FOR THE LARVAE OF THE CECROPIA MOTH, *SAMIA CECROPIA* (LINN.)

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## INTRODUCTION

Dethier (1937, 1939) and Eger (1937) have so far conducted the only studies on gustation in lepidopterous larvae. Dethier, interested in localizing the gustatory receptors and in gustatory acuity and its relation to host selection by caterpillars, has determined acceptance thresholds for certain sugars and rejection thresholds for hydrochloric acid for a number of species. Eger, interested chiefly in determining the modalities of taste for caterpillars, has determined acceptance thresholds for some sugars and rejection thresholds for hydrochloric acid, sodium chloride, acetic acid, quinine hydrochloride, and saccharine. He concludes that there are probably only two modalities for caterpillars—acceptable and unacceptable, but he admits that his data offer no definite proof for this. The following experiments were undertaken to add more information to that already existing in this little known field.

## MATERIALS AND METHODS

The larvae of *Samia cecropia* used in the following experiments were reared from a single group of eggs laid by a gravid female captured in the early summer, the food plant being the sycamore (*Platanus occidentalis*). They were at first confined in a large jar and fresh leaves were added as needed, but later they were put on individual branches and numbered by marking with drops of colored paint. All the animals used in the experiments described below were in the last instar.

Rejection thresholds were determined, without disturbing the larvae, by placing drops of test solutions on the leaves as the animals fed. Since the caterpillars eat leaves in a very orderly fashion, it is easy to place drops so that they are reached by them within a very short time, usually about 10 to 15 seconds. Drops were placed on the leaves from small, new and carefully washed artists' brushes kept in the test solutions. A definite attempt was made to have the drops as nearly the same size as possible, but the importance of this is doubtful, for the larvae ordinarily ate on, undisturbed, below their thresholds and reacted almost immediately upon contact when the solutions were concentrated enough to bring about rejection.

The test solutions were prepared from C.P. chemicals, unless otherwise noted, using volumetric apparatus, and all tests were run within three hours after the solutions were prepared. The following chemicals were used, the concentrations prepared being given in parentheses after each: sucrose (2M, 1M, 0.5M), glucose, U.S.P. (2M, 1M, 0.5M), lactose, U.S.P. (1M, 0.5M), strychnine sulfate, U.S.P. (saturated solution), hydrochloric acid (0.4N, 0.2N, 0.1N, 0.075N, 0.050N, 0.025N), acetic acid (0.8N, 0.4N, 0.2N, 0.1N, 0.075N, 0.050N), sodium hydroxide

(0.4N, 0.2N, 0.1N, 0.075N, 0.050N), sodium chloride (2N, 1N, 0.75N, 0.50N, 0.25N), lithium chloride (4N, 2N, 1N, 0.75N, 0.50N), calcium chloride (1N, 0.75N, 0.50N, 0.25N, 0.10N), ammonium chloride (1N, 0.75N, 0.50N, 0.25N, 0.10N), and potassium chloride (1N, 0.75N, 0.50N, 0.25N, 0.10N).

There was no particular order in which these solutions were presented to the individual larvae. Since tests could be carried on only during feeding, it was found convenient to use three or four solutions simultaneously with all the caterpillars, testing the animals, whenever they fed, with the various concentrations. Thus there was no chance for associations to be built up by the caterpillars through consistent use of ascending or descending orders of concentrations. Each of the thresholds was determined at least three times for each of the caterpillars, and, in most cases, was determined five or six times, the greatest number for any individual being nine times. Acceptances are easy to determine, because the animals eat three or four "cuts" through the drop in their feeding. Rejections are usually equally sharp, because the caterpillars stop feeding immediately upon touching the drop, withdraw and start feeding elsewhere. It was possible, therefore, although only twelve specimens were used, to make accurate determinations for these solutions under these conditions.

The temperature of the laboratory was 23° C. and the relative humidity 70 per cent throughout all the work. Ordinary daylight entering the windows was the source of illumination.

## RESULTS

There were no rejection thresholds for sugars or for strychnine sulfate, the larvae eating the most concentrated solutions of these offered to them. The thresholds for acids and salts used in these experiments are given in Table 1.

TABLE I

| Substance            | Range       | Mean           | $\frac{1}{\text{Threshold}}$ |
|----------------------|-------------|----------------|------------------------------|
| HCl                  | 0.04-0.15 N | 0.083 ± .008 N | 12                           |
| CH <sub>3</sub> COOH | 0.09-0.6 N  | 0.46 ± .05 N   |                              |
| NaOH                 | 0.09-0.15 N | 0.13 ± .008 N  | 7.7                          |
| KCl                  | 0.2 -0.9 N  | 0.45 ± .05 N   | 2.2                          |
| NH <sub>4</sub> Cl   | 0.4 -0.6 N  | 0.46 ± .03 N   | 2.2                          |
| NaCl                 | 0.4 -1.5 N  | 0.89 ± .1 N    | 1.1                          |
| CaCl <sub>2</sub>    | 0.2 -0.9 N  | 0.61 ± .05 N   | 1.6                          |
| LiCl                 | 0.9 -3.0 N  | 1.4 ± .15 N    | 0.72                         |

In the column headed, "Range," are the lowest and highest thresholds discovered for the group; this does not imply a uniform distribution between these two extremes, but it gives some indication of the individual variations. In the column headed, "Mean," are the mean threshold normalities and the standard errors of these means (probable errors would be .6745 times the standard errors). In the column headed, "1/Threshold," are the reciprocals of the thresholds, these figures to be used below as indicative of stimulative efficiencies.

Thresholds were calculated as follows. The two critical concentrations—that is, the pair of concentrations, the lower of which was accepted, the higher rejected—were determined for each larva. Obviously, the true threshold lies between these two, and, for statistical purposes, it was assumed that the threshold lay midway between them. Thus, if a larva accepted 0.25N and rejected 0.50N, the threshold assigned was 0.38N, if between 0.50N and 0.75N, the threshold assigned was 0.63N. Since the second digit cannot be interpreted literally in such a system, these thresholds are designated in the table as 0.4N and 0.6N respectively, only the means being given to the second place.

While the validity of using the midpoint between the two critical concentrations as the threshold for an individual animal can be questioned, certain supplementary observations and considerations made it appear permissible to use this for the calculation of means. Thus, most of the animals readily ate through the drop of lower concentration and stopped clearly on reaching the drop of higher concentration, indicating a threshold in the middle range between the two. A few, however, hesitated at the lower concentration before continuing with feeding and reacted violently at the higher, drawing back sharply and often ceasing feeding for a time, indicating a threshold near the lower concentration. Others ate readily through the lower concentration and only hesitated at first at the higher, made a few tries at eating it, but finally stopped and usually simply moved a short distance away on the same leaf, indicating a threshold near the higher concentration. Since these latter two cases seemed to be of equal occurrence, with the majority of rejections of the first type described, it seemed fair, for purposes of calculation of means for the group to be used for comparative purposes only, to assign the midpoints between the critical concentrations as the thresholds for the individuals, in the group, without implying that these represent true thresholds for any of the individuals.

## DISCUSSION

### *Sugars and strychnine sulfate*

Since there were no rejection thresholds for sugars or for strychnine sulfate, no conclusions can be drawn concerning the ability of the caterpillars to taste these substances. It must be recognized that failure of rejection does not imply that the animals cannot taste these compounds, just as rejection thresholds do not measure the lowest concentrations that can be tasted. To me, sycamore leaves tasted almost as bitter as the strychnine solution, and, since these larvae feed on many species of trees, it would seem logical that their rejection threshold for bitter substances would be high. Dethier (1937) has shown definitely that caterpillars of this species can taste glucose and sucrose, but he used acceptance thresholds in his work.

### *Acids*

The acid thresholds here given are higher than those given by Dethier and Eger, but that is to be expected, for the acids in their experiments were offered in drops of water, while in this case there was some mixing of the acids with the food. The pH of the solution of hydrochloric acid corresponding to the mean rejection threshold normality is 1.2, while the pH of the solution of acetic acid at the mean rejection threshold normality is 2.5. Thus, acetic acid, as for man, is much more

effective a stimulating agent than is hydrochloric acid at the same pH. The ratio for this species is 20:1, which compares favorably with Eger's (1937) report for another species of a ratio averaging about 23:1. This ratio is comparable to that for humans (28:1), and Eger suggests that it indicates a similarity in the buffering capacities of the salivas of the two species. It further, however, suggests a similarity of action in both, and may indicate a sensitivity to hydronium ion in these caterpillars corresponding to the sour taste in humans.

### *Salts*

The order of relative stimulative efficiencies, as measured by the reciprocals of the normalities of the rejection limens, for the various cations, as chlorides, gives the series:  $\text{NH}_4^+ = \text{K}^+ > \text{Ca}^{++} > \text{Na}^+ > \text{Li}^+$ .

This series can be verified further by checking the orders for the twelve individuals to determine in how many cases any specific cation has an equal, greater,

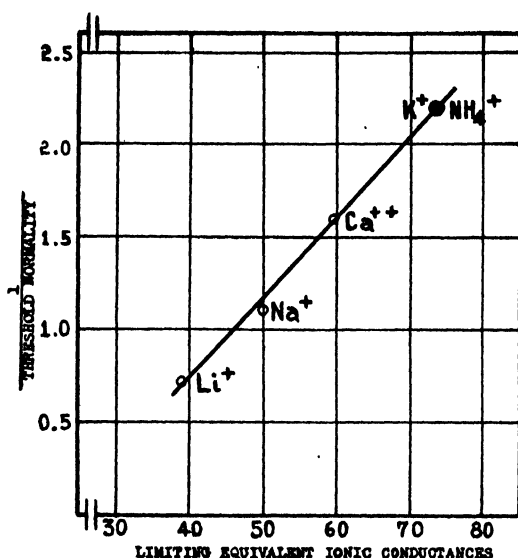


FIGURE 1. Showing the relationship between the stimulative efficiencies of the cations, as chlorides, and their limiting equivalent ionic conductances in Mhos at 25° C. Values for the latter are from Gucker and Meldrum (1942).

or lesser stimulative efficiency than any other cation. Using this method, it is found that  $\text{K}^+$  has a greater efficiency than  $\text{NH}_4^+$  ( $\text{K}^+ > \text{NH}_4^+$ ) in 4 individuals,  $\text{NH}_4^+$  has greater than  $\text{K}^+$  ( $\text{K}^+ < \text{NH}_4^+$ ) in 3 individuals, and  $\text{K}^+$  is equal to  $\text{NH}_4^+$  ( $\text{K}^+ = \text{NH}_4^+$ ) in 5 individuals. This indicates that  $\text{K}^+$  and  $\text{NH}_4^+$  are equal in stimulative efficiency. Comparing  $\text{K}^+$  and  $\text{NH}_4^+$  with  $\text{Na}^+$ , the following orders are noted:  $\text{K}^+ > \text{Na}^+ - 9$ ,  $\text{K}^+ = \text{Na}^+ - 3$ ,  $\text{K}^+ < \text{Na}^+ - 0$ , and  $\text{NH}_4^+ > \text{Na}^+ - 8$ ,  $\text{NH}_4^+ = \text{Na}^+ - 4$ ,  $\text{NH}_4^+ < \text{Na}^+ - 0$ . These clearly show that  $\text{K}^+$  and  $\text{NH}_4^+$  have greater stimulative efficiencies than  $\text{Na}^+$ , and further confirm their equality. Comparing  $\text{Na}^+$  with  $\text{Li}^+$ , the following are found:  $\text{Na}^+ > \text{Li}^+ - 9$ ,  $\text{Na}^+ = \text{Li}^+ - 3$ ,  $\text{Na}^+ < \text{Li}^+ - 0$ . Obviously,  $\text{Na}^+$  is more stimulating than  $\text{Li}^+$ .  $\text{K}^+$  and  $\text{NH}_4^+$  are

found to be more stimulating than  $\text{Li}^+$  in all cases, thus verifying their position above  $\text{Na}^+$ .  $\text{Ca}^{++}$  shows the greatest variation in position. Comparing with  $\text{NH}_4^+$  or  $\text{K}^+$ , the following are found:  $\text{K}^+$  or  $\text{NH}_4^+ > \text{Ca}^{++} - 8$ ,  $\text{K}^+$  or  $\text{NH}_4^+ = \text{Ca}^{++} - 2$ ,  $\text{K}^+$  or  $\text{NH}_4^+ < \text{Ca}^{++} - 2$ . Thus  $\text{K}^+$  and  $\text{NH}_4^+$  have greater stimulative effects than  $\text{Ca}^{++}$ . Comparing  $\text{Ca}^{++}$  with  $\text{Na}^+$  we find:  $\text{Ca}^{++} > \text{Na}^+ - 9$ ,  $\text{Ca}^{++} = \text{Na}^+ - 0$ ,  $\text{Ca}^{++} < \text{Na}^+ - 3$ .  $\text{Ca}^{++}$  is thus more stimulating than  $\text{Na}^+$ . With  $\text{Li}^+$  we find:  $\text{Ca}^{++} > \text{Li}^+ - 11$ ,  $\text{Ca}^{++} = \text{Li}^+ - 1$ ,  $\text{Ca}^{++} < \text{Li}^+ - 0$ ,  $\text{Ca}^{++}$  is thus more stimulating than  $\text{Li}^+$ . All of these individual orders considered together indicate the same arrangement as that found using the mean thresholds, namely:  $\text{NH}_4^+ = \text{K}^+ > \text{Ca}^{++} > \text{Na}^+ > \text{Li}^+$ .

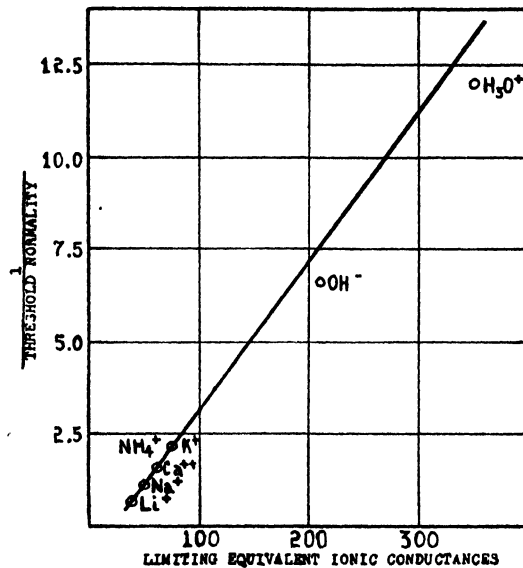


FIGURE 2. Showing the relationship between the stimulative efficiencies and the limiting equivalent ionic conductances in Mhos at  $25^\circ \text{C}$ . of certain ions. The slope is taken as determined by the metallic ions. Values for the conductances are from Gucker and Meldrum (1942).

Hopkins (1932) has tabulated the discoveries of numerous investigators concerning stimulative efficiencies of various cations and anions. The order shown in the case of the caterpillars of *Samia cecropia* here used is similar to that discovered for other species of animals. This suggests some basic chemical or physical reason for this order. Hopkins relates the order of cations for the oyster, which he studied, to the atomic weights and atomic mobilities. In the present case, there is no obvious relationship between ionic weights and stimulative efficiencies, but Figure 1 shows the close agreement between the stimulative efficiencies of the cations, as determined by the reciprocals of the normalities of the rejection limens, and their limiting equivalent ionic conductances which are directly proportional to the ionic mobilities, in fact, have sometimes been called mobilities. Since the chloride ion is the anion in all cases, it is impossible to determine its relative stimulative effect, and

it is, therefore, disregarded in this plot, since, whatever its efficiency, it is constant for all substances tested.

These relationships suggest a common mode of action, and possibly a common modality of taste, for all the cations tested. Since, however, this same order is found for man for  $K^+$ ,  $Na^+$ , and  $Li^+$ , and KCl has a taste easily distinguishable from NaCl or LiCl, this conclusion is not warranted without further evidence.

Sodium hydroxide shows a much greater stimulative efficiency than any of the other salts<sup>1</sup> used in this work, thus indicating that the  $OH^-$  ion is the critical one in its effectiveness, since some measure of the efficiency of the  $Na^+$  ion is given by the stimulative efficiency of NaCl. Comparison of the data for NaOH and HCl shows that the  $OH^-$  ion is much less stimulating than the  $H_3O^+$  ion.

Figure 2 shows the extrapolation of the graph, stimulative efficiency vs. limiting equivalent ionic conductance to include  $OH^-$  and  $H_3O^+$ . The value for the stimulative efficiency of the  $OH^-$  ion is approximated by subtracting from the stimulative efficiency for NaOH the stimulative efficiency for NaCl, assuming thus that most of this is due to the  $Na^+$  ion. This shows that there is a possibility that  $OH^-$  and  $H_3O^+$  fall in the same series. That they are apparently lower in stimulative efficiency than is needed for a perfect "fit" in the graph would be expected from the fact that the solutions containing them were mixed with the protoplasm of the leaves and with the saliva of the caterpillars, both of which are buffered. This might mean that there is only an "unacceptable" modality of taste for all these substances, as Eger (1937) suggests, but this cannot be decided without further evidence. The  $OH^-$  and  $H_3O^+$  ions, in the case of man, show a high stimulative efficiency when compared with other anions and cations, and stimulation by the  $H_3O^+$  ion, at least, is admitted to be the important factor in the sour taste, as opposed to the salt taste of the metallic chlorides.

In all, these results can only be taken as suggestive and indicative of a need for much more extensive and careful work on the gustatory responses of animals to more than the usual NaCl in testing the salt taste.

### SUMMARY

Rejection thresholds for HCl,  $CH_3COOH$ , NaOH, NaCl,  $NH_4Cl$ , KCl,  $CaCl_2$ , and LiCl, presented as drops of solutions on leaves of the food plant, were determined for caterpillars of the cecropia moth, *Samia cecropia*. Rejection thresholds for glucose, sucrose, lactose, and strychnine sulfate either do not exist under these conditions, or are higher than the saturation concentrations of solutions of these substances. The lowest threshold of those tested is that for HCl, but  $CH_3COOH$  has greater stimulative efficiency when it is compared with HCl at the same pH. The threshold for NaOH is higher than that for HCl, indicating that the  $OH^-$  ion is less stimulating than the  $H_3O^+$  ion. The order of stimulative efficiency for the cations, as chlorides, is  $NH_4^+ = K^+ > Ca^{++} > Na^+ > Li^+$ . This is the order of ionic mobilities to which the stimulative efficiencies seem to be related. No conclusions can be drawn with certainty as yet regarding the modalities of taste for these animals.

<sup>1</sup> Modern Acid-Base concepts require that NaOH be considered as a salt.

## ACKNOWLEDGEMENTS

It is a pleasure to express my appreciation for the many kindnesses shown me during this work by Dr. Nicholas Hyma, Head of the Department of Chemistry, and Dr. Arthur B. Gould, Professor of Chemistry, at this institution. An especial debt is due my wife who aided me in all phases of the work.

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# BACKGROUND ILLUMINATION AS A FACTOR IN THE ATTACHMENT OF BARNACLE CYPRIDS

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## INTRODUCTION

Various authors, following Visscher (1927), have shown that certain species of barnacles attach in greater numbers to darker surfaces. McDougall (1943), by means of an experiment designed to test the effect of varying degrees of illumination, demonstrated that larvae of *Balanus eburneus* have a tendency to settle most abundantly upon collectors placed in less brightly illuminated areas. Pomerat and Reiner (1942) showed that *Balanus eburneus* larvae attach more readily to black rather than to opal panels when they are exposed in the sea for several days. These investigators also observed that when light is at a minimum, during the hours of darkness, the differences do not occur but that attachments are remarkably similar on light and dark panels. This suggested that attachment to darker panels during daylight is a phototropic response to the contrasting effect of dark surfaces against lighter general surroundings. The purpose of the present study was to investigate whether in fact, contrasting surroundings are effective in promoting attachment. A further objective was the determination of the maximum distance at which the degree of illumination of a background surface is effective in influencing attachment to transparent surfaces.

The experiments were conducted at the Pensacola, Florida laboratory of the U. S. Fish and Wildlife Service by the kind permission of Dr. A. E. Hopkins during the summer months of June, July, and August, 1942. Acknowledgments are also due to Dr. C. M. Pomerat for his generous advice, and to Dr. F. G. Walton Smith, Director of the University of Miami Marine Laboratory, for assistance in preparing the paper and in evaluating the results.

## EFFECT OF CONTRASTING BACKGROUNDS

In the experiments designed to determine whether contrasting surroundings influence cyprid attachment, provision was made for base plates of black, transparent, and opal Carrara glass plates 10" x 12" in size. Upon each of these were mounted 4" x 10" black and opal Carrara glass collecting panels, in such a way as to be surrounded by a 2 inch border of the base panel.

The plates were supported by a wooden frame. The glass bases were arranged one inch apart and the paired black and opal collectors were separated by two inches. Thus, a series of contrasts was offered between the pairs of black and opal plates and the three surrounding borders of black, opal, and transparent glass. The barnacles were counted following attachment upon the collectors, each of which offered an exposed area of 36 sq.in. In the locality where the investigations

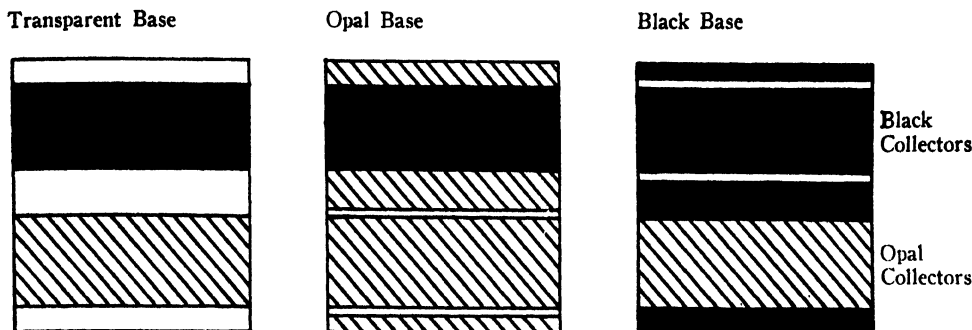


FIGURE 1. Arrangement of black and opal collectors upon black, opal, and transparent bases.

were carried out the adults of *Balanus cburneus* were the barnacles most commonly found, and all attachments were assumed to belong to this species.

The apparatus for the first series of experiments was lowered into the sea with the plates in a vertical position at a mean depth of three feet from the surface. Six successive experiments were conducted with an average duration of 36 hours. At the end of each period the paired plates were removed and washed gently with fresh water to remove salt crystals and silt. Counts were then made of the cyprids and of the metamorphosed barnacles, with the results shown in Table I.

Further experiments were conducted with the plates exposed horizontally, in order to collect attachments on the lower surface. The mean depth of the plates below the surface of the sea was seven feet. Five experiments were conducted,

TABLE I  
Barnacles attaching to vertical collectors with borders of varying contrast

| Experiment | Date of exposure | Black collector |      |       | Opal collector |      |       |
|------------|------------------|-----------------|------|-------|----------------|------|-------|
|            |                  | Borders         |      |       | Borders        |      |       |
|            |                  | Transparent     | Opal | Black | Transparent    | Opal | Black |
| I          | 7- 2-44          | 68              | 67   | 35    | 15             | 17   | 8     |
| II         | 7- 4-44          | 300             | 293  | 458   | 249            | 183  | 233   |
| III        | 7- 6-44          | 73              | 115  | 59    | 8              | 5    | 7     |
| IV         | 7- 8-44          | 120             | 175  | 121   | 15             | 38   | 35    |
| V          | 7-10-44          | 89              | 97   | 86    | 17             | 7    | 25    |
| VI         | 7-12-44          | 137             | 152  | 162   | 23             | 23   | 18    |
| Total      |                  | 787             | 899  | 921   | 327            | 273  | 326   |
| Average    |                  | 131             | 150  | 154   | 55             | 46   | 54    |

TABLE II

Barnacles attaching to horizontal collectors with borders of varying contrast

| Experiment | Date of exposure | Black collector |      |       | Opal collector |      |       |
|------------|------------------|-----------------|------|-------|----------------|------|-------|
|            |                  | Borders         |      |       | Borders        |      |       |
|            |                  | Transparent     | Opal | Black | Transparent    | Opal | Black |
| VII        | 7-17-44          | 653             | 503  | 703   | 80             | 114  | 171   |
| VIII       | 7-20-44          | 757             | 692  | 711   | 94             | 142  | 194   |
| IX         | 7-23-44          | 582             | 720  | 686   | 142            | 249  | 340   |
| X          | 7-26-44          | 620             | 855  | 701   | 254            | 328  | 437   |
| XI         | 7-31-44          | 526             | 582  | 564   | 194            | 230  | 303   |
| Total      |                  | 3138            | 3352 | 3365  | 764            | 1063 | 1445  |
| Average    |                  | 628             | 670  | 673   | 153            | 213  | 289   |

with exposure periods varying from 72 to 120 hours. The results are shown in Table II.

Upon horizontal and vertical black collectors and regardless of the nature of the surroundings, greater attachments occurred upon the black collectors as compared with the opal. The total number of organisms on all black horizontal collectors amounted to 9855 compared with a total of 3272 for opal horizontal collectors, giving a black/opal distribution ratio of 3.0. The total populations on black and opal vertical collectors were 2607 and 926 respectively, giving a ratio of 2.8. These ratios, though greater, qualitatively substantiate the findings of Pomerat and Reiner (1942) whose ratio for attachment numbers upon black and opal collectors was 1.8 (Table III).

TABLE III

Total number of barnacles on black and opal collectors

|            | Black collectors | Opal collectors | Ratio Black collectors<br>Opal collectors |
|------------|------------------|-----------------|---|
| Horizontal | 9855             | 3272            | 3.0                                       |
| Vertical   | 2607             | 926             | 2.8                                       |

Attachments to black vertical collectors did not vary greatly with the type of surrounding, but were least in the case of the transparent border (Table I). With black collectors, borders of black, transparent and opal, in this order, afford increasing contrasts. Had the contrast been effective, greater attachments should have occurred upon collectors with the opal and transparent borders rather than with the black border. Attachments upon black horizontal collectors also failed to

show significant differences when the three different surroundings were employed, although they were least in the case of a transparent border (Table II).

The frequencies of attachment upon vertical opal collectors did not show any correlation with increasing degree of contrast but were similar in all instances (Table I). The horizontal opal collectors gave somewhat different results (Table II). In this series of experiments the greatest attachments were found on the collectors with the black borders. The least numbers, however, were observed on collectors with transparent borders and intermediate attachment frequency occurred with opal borders. In these experiments the least degree of contrast was offered by opal surroundings of similar material to the collectors and the greatest degree by black surroundings which afforded the minimum possibilities of transmitted and reflected light. There was not, therefore, a consistent correlation between the intensity of attachment and the degree of contrast between collector and surroundings.

In an attempt to explain the results, the question of variation in the general intensity of illumination beneath the panels may be considered. The amount of transmitted and reflected light was greatest around horizontal opal collectors with transparent surroundings. The reflected light which alone fell below opal collectors with the opal borders was less intense than the light in the former case and it was under this condition that frequency of attachment was found to be increased. The opal collectors with black borders, which reflected the least light of the three types of surrounding were found to have the greatest attachment. Thus, with the decrease in general illumination beneath opal collectors, there was an increase in attachments, and it appears that attachment frequencies are here related to the degree of shading under the collectors caused by the varying opacity and reflection of the surroundings.

The horizontal black collectors were found to have distinctly greater attachments when combined with a black border. They also showed least attachments with a clear border. The black collectors, therefore showed a correlation, not with contrast but rather with the degree of shading which is at a maximum where both collector and surroundings are black.

In the case of both black and opal vertical collectors, the shading effect of the border is less pronounced, due to the fact that much of the light enters the water at a more or less vertical angle. The hypothesis that general shading stimulates attachment would fit in with the observed facts about attachments to these series. The difference between the amount of light reflected by black or opal collectors is more significant than that reflected by the relatively small borders, and hence accounts for the relatively small differences among attachments to black collectors with different surroundings or among attachments to opal collectors with varied surroundings. The differences between attachments to the two types of collector with each surrounding are relatively greater.

#### DISTANCE AT WHICH BACKGROUND ILLUMINATION IS EFFECTIVE

The equipment designed for the question of determining the maximum distance at which black or opal surfaces are effective in stimulating a cyprid to attach consisted of a wooden frame 30" long, 32" wide, and 12" high. This frame supported vertically two transparent glass plates. Behind the first glass plate was mounted a

black plastic panel in such a manner that it might be moved to varying distances. The second transparent glass plate without any backing was used as the control. A second series of transparent glass plates at the opposite side of the frame were treated in the same manner with the exception that an opal plastic panel was used instead of a black one. The movable black plastic panel and the movable opal plastic panel were set at increasing distances from the clear panel for successive experiments. Each transparent glass plate from which the barnacles were counted, offered 54 sq.in. of surface area. They were exposed at a mean depth of three feet from the surface for an average duration of 72 hours. In the successive experiments the black and opal plastic movable panels were placed at 2", 3", 4", and 6" behind the transparent collectors.

The results (Table IV) show a general decline in the attachments to the glass collector as the black movable plate was moved away. The maximum distance at

TABLE IV  
Attachment of barnacles to transparent panels with movable backgrounds

| Distance of background from collector | Movable black | Control transparent | Ratio Movable Control | Movable opal | Control transparent | Ratio Movable Control |
|---------------------------------------|---------------|---------------------|-----------------------|--------------|---------------------|-----------------------|
| 2 inches                              | 2998          | 778                 | 3.9                   | 1258         | 511                 | 2.5                   |
| 3 inches                              | 803           | 355                 | 2.3                   | 835          | 336                 | 2.5                   |
| 4 inches                              | 3759          | 1707                | 2.2                   | 3686         | 2806                | 1.3                   |
| 6 inches                              | 458           | 453                 | 1.0                   | 906          | 780                 | 1.2                   |

which it influenced attachment, as determined by comparison with the control, was between four and six inches. The same general decline in population was noted for the opal plates although the maximum influencing range was greater than six inches. Since attachment is influenced by the movable panels at a distance, it is obvious that the light reflection at the actual collecting surface is not the principal stimulating factor. The facts are more readily explained by assuming that the intensity of general illumination in the vicinity of the collector is the important factor and that where this is reduced by the proximity of black backplates greater attachment occurs. Decreasing distances of the opal backplate, while giving a superficial appearance of increased light intensity, probably succeed in blocking the light passing through the collecting panel. Thus increased attachment under these conditions may still be due to decreased general illumination.

These general conclusions agree with the results of the contrasting background experiments described in the first part of the paper where a relationship appeared to exist between the number of attachments and the amount of shading caused by the type of background employed. The decreased intensity of illumination in the general vicinity of the collector, as opposed to the conditions of illumination immediately upon the collector itself may increase attachment by causing a response in the cyprid. This response is in the nature of a conditioning towards subsequent attachment, or the development of a physiological state favoring a subsequent

attachment response to a contact stimulus. The possibility of a tropism is not supported by the contrast experiments.

In the light of the above conclusions it may be interesting to recall the experiments of Schallek (1943) and Whitney (1941) who maintain that directional light is not to any great degree present under natural aquatic conditions but that conditions of diffuse light almost invariably predominate. This diffuse light is a naturally occurring phenomenon, whereas light from any one direction may not predominate sufficiently for a tropism to occur.

#### SUMMARY

1. Experiments were conducted to determine the effect of contrasting surroundings upon the frequency of attachment of *Balanus eburneus* larvae to opaque black and opal glass collecting surfaces. Further experiments were carried out to determine to what extent the number of attachments of transparent collectors was influenced by black and opal backgrounds placed at varying distances.

2. Greater numbers of attachments occurred upon the under side of horizontal rather than vertical, and upon black rather than opal collectors, thus confirming the observations of previous authors.

3. No correlation was found between the degree of contrast shown in the collector and surroundings, and the frequency of attachments.

4. Both black and opal surfaces were found to increase frequency of attachment when placed behind transparent collectors up to distances of six inches.

5. A definite dependence was found to exist between the frequency of attachment and a decrease in the intensity of general illumination in the area immediately beneath horizontal opal collectors. Similarly, the influence of movable backgrounds appeared to be in the nature of a shadow effect. It is suggested that "shading" acts as a stimulus which brings about favorable physiological conditions for the subsequent attachment of barnacle larvae and that the amount of light reflected from the collecting surface is only important insofar as it affects the general "shading" in the vicinity.

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## THE CONDITIONS THAT LEAD TO NORMAL OR ABNORMAL DEVELOPMENT OF CIONA

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In earlier papers I have discussed the problem of normal versus abnormal development of cross-fertilized eggs of *Ciona*. It was shown that external conditions sometimes influence the result; at other times it was not apparent that the abnormal development was due to such conditions. However, by a critical series of experiments evidence was found that the eggs are very sensitive to what may be called the "cleanliness" of the dishes, i.e. to either chemical or organic contamination. Further experiments have been made to find out whether internal factors may also be concerned with abnormal development of the eggs.

*Ciona* is a particularly favorable type for study of this problem. The eggs, as soon as they are mature, leave the ovary and accumulate for 24 hours or longer in the oviduct. The germinal vesicle has disappeared and the polar spindle comes to lie at the pole of the egg. The polar bodies are not given off until a spermatozoon enters the egg, i.e. after the eggs have been set free in the sea water. This may happen every 24 hours or be delayed for several days. Spawning occurs early in the morning. If not ejected at the regular time the eggs may accumulate in the oviduct in very large numbers. When the animals are brought into the laboratory and kept in running sea water, or in sea water that is aerated by a stream of air bubbles, the *Cionas* may hold back their eggs for several days. It has been shown that these delayed eggs may give rise to normal embryos, but it has not been shown clearly that the percentage of abnormals may not be increased. Fresh eggs removed from the oviduct and kept for as long as 24 hours in covered dishes of shallow sea water can be cross fertilized and may give normal embryos.

The sperm are matured in the tubes of the testes that ramify over the walls of the intestine in the region of the outlet of the ovary. The ripe sperms pass into the long sperm duct that runs parallel to the oviduct. Only ripe sperms are present in the sperm duct, and some of them are set free when the eggs are ejected. When the eggs are collected by puncturing the oviduct, only mature eggs are set free, and likewise only ripe sperm comes out when the sperm duct is cut across. In most other marine animals, whose eggs are used for experimental purposes, one is apt to get ripe and unripe eggs when the ovary is opened. The uniformity of the eggs from the oviduct of *Ciona* simplifies the experiment.

In another respect, also, *Ciona* offers favorable material for a study of normal versus abnormal development. It is well known that polyspermy often plays a significant note in laboratory experiments, unless it is carefully guarded against. In *Ciona*, polyspermy also occurs after cross-fertilization, but is very rare and will account for only a small percentage of abnormality. It can easily be detected at the two-cell stage when the egg divides into four or more parts if polyspermic.

Even in cases where much abnormality occurs, I have found, by watching the cleavage, that polyspermy was not the main agent in the results.

Concerning the kind of abnormalities that occur, not much can be said. For convenience I have recognized three classes; (1) Normals, (2) Abnormals, and (3) Bent (or twisted) embryos. The abnormals are those that have not gone beyond an early stage of development. They are usually in the egg membrane and are spherical with one or two black "eye" specks. The twisted or bent are later stages in development, usually out of the egg membrane. The tail is present and crooked or bent, sometimes still coiled loosely or irregularly about the body. There are overlaps between the three different types. All three are generally found in the same dish, but the external or internal conditions that produce each kind are not known. It is safe to assume, however, that polyspermic eggs do not pass beyond the earliest stages of development, but external conditions may also stop the development in early stages.

*Normal and Abnormal Development in Reciprocal Crosses*

To test to what extent the ratios of normal to abnormal development are due to intrinsic (genetic?) factors or due to environmental conditions, further experiments were made in May 1944. Not only were reciprocal crosses made, but samples of the same eggs were crossed with three different sperm suspensions.

TABLE I

The percentage of normal embryos resulting from reciprocal crosses. The small numbers under the percentages represent, from left to right, the numbers of normal, bent, and abnormal embryos. The bent embryos have been left out of account in calculating the percentages.

| <i>c sp</i> | <i>b sp</i> | <i>a sp</i> |               |
|-------------|-------------|-------------|---------------|
| 0           | 93          | 8           |               |
| 0 0 148     | 126 0 8     | 12 42 132   | <i>D eggs</i> |
| 70          | 89          | 99          |               |
| 67 9 28     | 49 2 6      | 128 0 1     | <i>E eggs</i> |
| 59          | 96          | 49          |               |
| 73 33 49    | 310 1 12    | 51 7 54     | <i>F eggs</i> |
| <i>f sp</i> | <i>e sp</i> | <i>d sp</i> |               |
| 0           | 3           | 52          |               |
| 0 0 37      | 1 2 28      | 10 22 9     | <i>A eggs</i> |
| 14          | 2           | 72          |               |
| 10 12 62    | 1 0 37      | 43 11 16    | <i>B eggs</i> |
| 16          | 10          | 0           |               |
| 22 29 116   | 11 23 96    | 0 0 177     | <i>C eggs</i> |

Some of the eggs of A were distributed in three dishes (horizontal lines, Table I), also some of the eggs of B and C, each to three dishes. These were cross-fertilized with sperm of d, e and f. Two drops of sperm were added to each dish, and the normal, bent, and abnormal embryos recorded after 24 hours.

The eggs of D, E, and F had been put into three dishes each (horizontal lines) before the sperm of the same animals, d, e, and f, had been removed to cross-

fertilize the eggs of A, B, and C. Then two drops of the sperm suspension of a, b, and c were added to the D, E, and F eggs. These crosses are the reciprocals of the preceding ones.

In calculating the percentages, the bent embryos were left out of account. Most of them were so nearly fully developed that they might be classified with the normal, rather than with the abnormal. The bent embryos overlapped those abnormal embryos that were fairly well developed. The bent embryos would not have affected the percentages to any great extent if half of them had been combined with the normals and half with the abnormal.

As shown in Table I the percentages of normal embryos resulting from crossing A eggs by d sperm, e sperm and f sperm are very different (52, 3, 0%). This holds also for B eggs and C eggs by the same sperm. In other words, the results are different for the different combinations, and not consistent either for the eggs or for the sperm.

The reciprocal crosses are shown above in the same Table I. Comparing them, there is seen to be no agreement in the percentages. In other words, the reciprocals are no more likely to be alike than other crosses. Before discussing these results, another similar experiment will be described that was made the next day with Cionas brought to Pasadena (Table II).

This experiment was carried out in the same way as the last, except that in addition to the reciprocals, three more dishes of A, B, and C eggs were set out and crossed with sperm from three other Cionas, viz. g, h, and i. These serve to give further data for the same eggs with different sperm. The reciprocals of g, h, i were not made. Comparing the reciprocals of the first three tests, D, E, and F, each set are more like each other than are the sperm tests a, b, and c, and also more like each other than in the first experiment, but still not the same inter se. The F eggs were much fewer than the others and produced relatively much smaller numbers of normals.

A comparison of the three kinds of eggs, A, B, and C each with the six kinds of sperm, d-i, shows that there are wide differences in the percentages (Table II). Since the environment was as nearly the same as possible, it may seem to follow that the differences are due to internal factors of some kind, possibly genetic in origin.

Why are the reciprocals so often different? The diploid eggs are presumably all genetically alike in each Ciona before fertilization, but after the polar bodies are extruded, the eggs become haploid, and should give as many kinds of eggs as there are combinations of the six pairs of chromosomes (excluding the possibility of a larger number due to crossing over). If amongst these combinations there are certain combinations that might give abnormal development—lethals, in fact,—it might seem that the chances are about the same in the cross and in its reciprocal. In other words, even with many lethal factor combinations, any differences in reciprocal ratios would only be due to chance, unless the cytoplasm of the diploid egg is involved in the situation.

There are several more or less probable hypotheses that suggest themselves, and some of these may or may not have some connection with the theory of self-sterility in Ciona; for, if certain classes should be eliminated, those that remain and produce normal tadpoles might, theoretically, be the ones that carry factors for self-sterility.

An examination of the data from the dishes of the same eggs (horizontal lines; Table II), each fertilized by different sperm, show very great differences in most cases in the percentages of normals to abnormals. Occasionally, however, a set gives very low values (Table I, F eggs by (a), (b), (c) sperm) or very high ratios (Table II, E eggs by (a), (b), (c) sperm. Also an examination of the ratios of the same sperm used to fertilize three different lots of eggs shows sometimes high percentages in all three dishes (Table II, (d) sperm by A, B, C eggs) and at other times low percentages in all three dishes (Table II, (g) sperm by A, B, C eggs). On the other hand there are cases where each of the three sets of eggs, and also each of the three sets of sperm, show great differences in the ratios.

If the data are significant, i.e., if the differences are not due to infection in the

TABLE II

The percentages of normal embryos resulting from reciprocal and one way crosses. The small numbers under the percentages represent, from left to right, the numbers of normal, bent, and abnormal embryos. The bent embryos have been left out of account in calculating the percentages.

|             |             |             | <i>c sp</i> |      | <i>b sp</i> |      | <i>a sp</i> |      |               |
|-------------|-------------|-------------|-------------|------|-------------|------|-------------|------|---------------|
|             |             |             | 97          |      | 76          |      | 91          |      |               |
|             |             |             | 71          | 2 1  | 287         | 8 35 | 152         | 3 14 | <i>D eggs</i> |
|             |             |             | 98          |      | 95          |      | 98          |      |               |
|             |             |             | 303         | 12 6 | 256         | 3 9  | 289         | 11 5 | <i>E eggs</i> |
|             |             |             | 3           |      | 2           |      | 0           |      |               |
|             |             |             | 1           | 0 32 | 1           | 0 48 | 0           | 0 34 | <i>F eggs</i> |
| <i>i sp</i> | <i>h sp</i> | <i>g sp</i> | <i>f sp</i> |      | <i>e sp</i> |      | <i>d sp</i> |      |               |
| 99          | 37          | 3           | 99          |      | 96          |      | 82          |      |               |
| 319         | 0 3         | 69          | 34          | 118  | 8           | 17   | 258         | 236  | 1 1           |
|             |             |             |             |      |             |      |             |      | <i>A eggs</i> |
| 2.8         | 0           | 0.2         | 76          |      | 58          |      | 97          |      |               |
| 9           | 65 280      | 0           | 8           | 286  | 1           | 0    | 403         | 360  | 72 112        |
|             |             |             |             |      |             |      |             |      | <i>B eggs</i> |
| 0           | 50          | 12          | 47          |      | 91          |      | 88          |      |               |
| 0           | 0 101       | 36          | 32          | 35   | 1           | 8    | 7           | 200  | 1 24          |
|             |             |             |             |      |             |      |             |      | <i>C eggs</i> |

dishes, these results show that the outcome is due neither to the eggs alone nor to the sperm alone, but to the combinations found at the time of fertilization.

In this connection there are some further considerations to be taken into account. The cytoplasm of the eggs has developed under the influence of the diploid set of chromosomes. If the constitution of this cytoplasm determines whether the development will be normal or abnormal, then the eggs of one individual might be expected to develop in the same way irrespective of what kind of sperm fertilizes the eggs. On the other hand, after the extrusion of the polar bodies, the haploid egg nucleus may have a very different make-up from the original diploid nucleus. But it is not likely that this haploid nucleus can affect or change the constitution of the cytoplasm in the very short time before it combines with the sperm nucleus to form a new diploid nucleus, whose products carry on throughout the following development. From the evidence discussed above it might seem, therefore, that whether the development is normal or abnormal will depend on whether the

combination of the two haploid genetic chromosome groups are harmonious or not in their action on the cytoplasm. If the cytoplasm is indifferent, then reciprocal crosses should give the same ratios, but since they do not do so, it may seem to follow that the cytoplasm, which has developed under the influence of the diploid egg nucleus, is one of the factors in determining whether normal or abnormal development takes place. This conclusion would mean that certain kinds of combinations are being continually eliminated. But it would not necessarily mean that these have anything to do with cross and self-fertilization of the combinations that survive. However, experiments that will be described later make it probable that the differences in the dishes reviewed in this experiment are due to external factors, such as toxic infection, and are not due to inherited cytoplasmic differences in the eggs.

#### *Test for Polyspermy and Abnormal Development*

Five new sets of experiments were made (Table III) to test whether the abnormalities that appear frequently are due to polyspermy, although it had often been recorded that the division of the eggs at once into four (or more) cells only very rarely occurs. Forty, 20 or 2 drops of sperm suspension were added to eggs in Syracuse dishes. After one hour the supernatant fluid was changed to fresh sea water. Reciprocal crosses were made in all cases. As the table shows, Table III, the relative number of normals to abnormalities was no lower after 40 drops than after 2 drops of sperm suspension. Evidently polyspermy is not the cause of abnormal development. There are several striking exceptions to the general results. One (June 18-19, 1944),  $A^1-b^1$ , gave after 2 drops almost all abnormalities (328), although after 40 or 20 drops nearly all were normal. Again (June 19-20,  $A^2-b^2$ , in one case after 20 drops all were abnormal (295); and in the reciprocal after 40 drops all were abnormal (199), although after 20 and 2 drops nearly all were normal. In another test (June 18-19),  $A^3-b^3$ , normals greatly predominated. In another (June 18-19),  $A^4-b^4$ , all were abnormal after 20 drops, but normal after 40 and after 2 drops sperm suspension. In another lot (June 17-18),  $A^5-b^5$  and  $B^5-a^5$ , there were two cases where there were more abnormalities than normals. These apparently contradictory results can only be explained on the assumption that the dishes were in some way responsible for the abnormal developments.

#### *Delay in Fertilization and Abnormal Development*

Whether or not a delay in fertilization causes abnormality after eggs and sperm have been kept in sea water was tested. The results are recorded in Table IV. The eggs of three individuals were distributed amongst six dishes. Then the eggs of A were fertilized by 10 drops of the sperm suspension of b and c at once, and after an hour and two hours. Similarly the eggs of B were fertilized by the sperm of a and c; and for the eggs of C by the sperm of a and b.

In A by b normals were produced by fertilizing at once, and after one hour, but nearly all abnormalities after two hours. This is true also for A by c, but B by a and B by c and C by b gave more normals after two hours. C by a gave normals after one hour, but no fertilization after 2 hours. Perhaps the most striking results are A by b and A by c that gave normals at once and after a delay of one hour, but abnormalities after two hours, but this did not happen in two other cases.

Either the sperm in the two latter cases was less affected by the delay, or there was some kind of difference in the dishes themselves. The irregularities in this experiment could only be accounted for on the assumption of infection in some of the dishes. The test was repeated (Oct. 14, 1944) with the eggs of new Cionas at

TABLE III

The effect of density of insemination in reciprocal crosses on the proportions of normal tadpoles, bent embryos, and abnormal embryos.

(June 18-19, 1944)

| A <sup>1</sup> by b <sup>1</sup> | Tad | Bent | Abn. | B <sup>1</sup> by a <sup>1</sup> | Tad | Bent | Abn. |
|----------------------------------|-----|------|------|----------------------------------|-----|------|------|
| 40 drops                         | 405 | 0    | 0    | 40 drops                         | 406 | 6    | 17   |
| 20 drops                         | 443 | 0    | 2    | 20 drops                         | 471 | 3    | 13   |
| 2 drops                          | 3   | 40   | 328  | 2 drops                          | 364 | 8    | 4    |

(June 19-20, 1944)

| A <sup>2</sup> by b <sup>2</sup> | Tad | Bent | Abn. | B <sup>2</sup> by a <sup>2</sup> | Tad | Bent | Abn. |
|----------------------------------|-----|------|------|----------------------------------|-----|------|------|
| 40 drops                         | 122 | 21   | 39   | 40 drops                         | 0   | 0    | 199  |
| 20 drops                         | 0   | 0    | 295  | 20 drops                         | 149 | 12   | 7    |
| 2 drops                          | 351 | 0    | 0    | 2 drops                          | 91  | 0    | 0    |

(June 18-19, 1944)

| A <sup>3</sup> by b <sup>3</sup> | Tad | Bent | Abn. | B <sup>3</sup> by a <sup>3</sup> | Tad | Bent | Abn. |
|----------------------------------|-----|------|------|----------------------------------|-----|------|------|
| 40 drops                         | 211 | 5    | 0    | 40 drops                         | 205 | 10   | 21   |
| 20 drops                         | 268 | 7    | 17   | 20 drops                         | 101 | 23   | 26   |
| 2 drops                          | 148 | 14   | 7    | 2 drops                          | 231 | 1    | 3    |

(June 18-19, 1944)

| A <sup>4</sup> by b <sup>4</sup> | Tad | Bent | Abn. | B <sup>4</sup> by a <sup>4</sup> | Tad | Bent | Abn. |
|----------------------------------|-----|------|------|----------------------------------|-----|------|------|
| 40 drops                         | 230 | 0    | 0    | 40 drops                         | 225 | 24   | 37   |
| 20 drops                         | 0   | 0    | 263  | 20 drops                         | 330 | 0    | 1    |
| 2 drops                          | 266 | 0    | 0    | 2 drops                          | 218 | 19   | 38   |

(June 17-18, 1944)

| A <sup>5</sup> by b <sup>5</sup> | Tad | Bent | Abn. | B <sup>5</sup> by a <sup>5</sup> | Tad | Bent | Abn. |
|----------------------------------|-----|------|------|----------------------------------|-----|------|------|
| 20 drops                         | 377 | 6    | 7    | 20 drops                         | 607 | 13   | 31   |
| 10 drops                         | 293 | 3    | 1    | 10 drops                         | 684 | 2    | 12   |
| 2 drops                          | 45  | 43   | 116  | 2 drops                          | 191 | 35   | 278  |

Corona Del Mar, and transferred to Pasadena in autoclaved flasks and stenders. Five of the lots fertilized at once, A by b, gave 99.9 per cent normals; one (B by c) gave 75 per cent normals. After 2 hours A by b gave 99.9; A by c, one normal and the rest unfertilized; A by c gave 0.5 per cent normal and the rest unfertilized.

TABLE IV

The effect of delaying insemination in reciprocal crosses on the proportions of normal, bent, and abnormal embryos, and unfertilized eggs.

|        |         | N   | Bent | Abn. | Unfert. |        |         | N   | Bent | Abn. | Unfert. |
|--------|---------|-----|------|------|---------|--------|---------|-----|------|------|---------|
| A by b | at once | 504 | 0    | 7    | 6       | B by a | at once | 66  | 13   | 232  | 93      |
|        | 1 hr.   | 343 | 0    | 1    | 0       |        | 1 hr.   | 131 | 2    | 84   | 98      |
|        | 2 hrs.  | 3   | 5    | 390  | 25      |        | 2 hrs.  | 204 | 0    | 0    | 179     |
| A by c | at once | 428 | 0    | 20   | 27      | C by a | at once | 317 | 7    | 48   | 11      |
|        | 1 hr.   | 464 | 0    | 9    | 27      |        | 1 hr.   | 304 | 1    | 11   | 12      |
|        | 2 hrs.  | 14  | 30   | 364  | 4       |        | 2 hrs.  | 0   | 0    | 0    | 498     |
| B by c | at once | 763 | 8    | 39   | 20      | C by b | at once | 721 | 0    | 15   | 5       |
|        | 1 hr.   | 483 | 14   | 63   | 4       |        | 1 hr.   | 870 | 0    | 8    | 6       |
|        | 2 hrs.  | 888 | 2    | 10   | 7       |        | 2 hrs.  | 910 | 1    | 1    | 157     |

After 3 hours A by b gave 95 per cent normals and 5 per cent unfertilized; A by c gave 99 per cent normals and 1 per cent unfertilized; B by c were all unfertilized. Reciprocally B by a at once was, by oversight, not fertilized; but after 2 hours 99.8 per cent, and after 3 hours 100 per cent were normals. C by a at once gave 99.9 per cent normals; after 2 hours 99.9 per cent normals; and after 3 hours 100 per cent normals. C by b at once gave 100 per cent normals; after two hours 99.9; and after three hours none were fertilized.

Another test of the same sort gave much the same kind of result. The dishes had been boiled. The percentages of normals was not so high, but there was no evidence of more abnormalities after one or 2 hours.

These tests make it quite clear that delay in fertilization up to 3 hours does not in itself cause abnormal development. The irregularities shown in Table IV may safely be ascribed to infection of the dishes. In fact other tests have shown that eggs may be kept in sea water for 24 hours, and, if fertilized with fresh sperm produce normal tadpoles.

#### *Eggs Fertilized En Masse and Distributed at Two-Cell Stage to Separate Small Dishes*

The exceptional cases, in which most or all of the eggs developed abnormally whereas others treated in the same way developed normally, suggested that some environmental conditions were responsible for the abnormalities. The following experiments were made to further test this suggestion. Eggs were collected from the oviduct in large clusters, and then drawn up by a pipette and transferred to finger bowls with about 80 cc. sea water. They were cross-fertilized with 10 to 20 drops of sperm suspension. After about one hour, when the two-cell stage was reached, about 100 were picked out with a pipette and put into several small dishes or flasks where they remained for about 24 hours, and were then classified. The eggs in the two-cell stage should be nearly alike on the whole, and any difference observed should be environmental. As shown in Table V, there were found some dishes that gave a high percentage of abnormalities. It would seem to follow that the differences are due to the environment rather than to internal factors. It is

especially to be noted that the eggs left in the finger bowls with more water generally gave nearly 100 per cent normals. The larger volume of water would be expected to dilute any contamination present in the larger dish or introduced with the eggs.

Again, a few eggs (about 100) in the two-cell stage were picked up with a pipette (2 or 3 drops) and each lot put into one of five small flasks, and into five jars with 10 cc. sea water. The flasks were stoppered and the jars had screw tops put on them to prevent evaporation. After twenty hours a few drops of formalin were added to each, and the condition of the embryos examined. Similarly the eggs of individual B were crossed by sperm of a. No counts were made

TABLE V

The effect of different glassware on the proportion of normal, bent, and abnormal embryos. Each of the two reciprocal crosses was made in a single finger bowl, and eggs in the two cell stage were pipetted into separate small dishes or flasks, in which they remained for about 24 hours.

| A by b | N   | Bent                | Ab  | B by a | N   | Bent         | Ab         |
|--------|-----|---------------------|-----|--------|-----|--------------|------------|
| 1      | 101 | 0                   | 3   | 1      | 0   | 0            | 271        |
| 2      | 103 | 0                   | 0   | 2      | 150 | 0            | 0          |
| 3      | 88  | 2                   | 6   | 3      | 0   | 0            | 176        |
| 4      | 164 | 2                   | 2   | 4      | 125 | 1            | 1          |
| 5      | 29  | short<br>62<br>bent | 4   | 5      | 12  | 56           | 93         |
| 6      | 15  | 3                   | 123 | 6      | 291 | 2            | 5          |
| 7      | 123 | 7                   | 5   | 7      | 75  | 12           | 0          |
| 8      | 0   | 0                   | 165 | 8      | 197 | 6            | 4          |
| 9      | 100 | 1                   | 6   | 9      | 4   | short<br>121 | 0          |
| 10     | 148 | 0                   | 4   | 10     | 8   | short<br>68  | bent<br>14 |

of the 20 lots since practically all dishes contained either 99 or 100 per cent normal tadpoles, except one flask that had 38 tadpoles, 48 bent tadpoles, and 21 abnormal embryos. There was no evident differences between this dish and the others.

Another experiment, similar to the last, with fresh Cionas was made (Aug. 12, 1944). Five Syracuse dishes (12 cc.) were used for some eggs, 5 Stender dishes for other eggs, and 5 small flasks for others. The dishes were left at Corona del Mar, and formalin added to each after 20 hours. Reciprocal crosses B by a were made. The fertilized eggs, not used, were kept in finger bowls (covered) in about 80 cc. sea water. A by b in the finger bowls gave 95 per cent normals and 5 per cent abnormals. B by a gave 99 per cent normals. The records of the smaller dishes are given in Table VI. It is noticeable that while normals greatly predominated, especially in B by a, there are three striking exceptions where abnormals predominate, and other where a good many abnormals were present. Since the controls—the original larger finger bowls—gave 95 and 98 per cent normals, the exceptional cases in the smaller dishes must have been due to contamination of some kind.

TABLE VI

The effect of different glassware on the proportion of normal, bent, and abnormal embryos resulting from two reciprocal crosses.

|          | A by b |     |      |     | B by a |      |     |
|----------|--------|-----|------|-----|--------|------|-----|
|          |        | N   | Bent | Ab  | N      | Bent | Abn |
| Syracuse | 1      | 0   | 20   | 213 | 61     | 30   | 93  |
|          | 2      | 121 | 9    | 12  | 115    | 22   | 10  |
|          | 3      | 112 | 5    | 19  | 52     | 16   | 84  |
|          | 4      | 100 | 24   | 6   | 157    | 0    | 7   |
|          | 5      | 87  | 2    | 7   | 107    | 15   | 12  |
| Stender  | 1      | 51  | 15   | 44  | 56     | 3    | 5   |
|          | 2      | 80  | 26   | 24  | 188    | 0    | 1   |
|          | 3      | 1   | 29   | 152 | 132    | 2    | 4   |
|          | 4      | 81  | 2    | 15  | 134    | 1    | 3   |
|          | 5      | 142 | 8    | 25  | 128    | 6    | 24  |
| Flask    | 1      | 58  | 7    | 11  | 35     | 0    | 1   |
|          | 2      | 89  | 2    | 14  | 77     | —    | 6   |
|          | 3      | 193 | 1    | 12  | 53     | 2    | 5   |
|          | 4      | 72  | 3    | 17  | 86     | 1    | 0   |
|          | 5      | 71  | 11   | 29  | 123    | 1    | 4   |

Another set of 20 tests was made with Syracuse dishes. The Cionas had been collected the day before and brought to Pasadena. They were in excellent condition with many stored eggs and much sperm. The eggs were inseminated with 30 drops of sperm in finger bowls of 80 cc. sea water. At the two-cell stage about 100 eggs were transferred to Syracuse dishes. Most of the dishes contained a high percentage of normals, but there were six dishes that contained nearly all abnormalities, and one that contained a large excess of bent and short embryos.

In the same lot, there were three dishes of A by b left uncovered that gave 12 bent and 111 abnormal embryos; one half covered that gave 3 normals, and 15 bent; and one  $\frac{7}{8}$  covered that gave 153 normals and 1 abnormal. The three similar reciprocals, B by c, gave nearly all normals. Since evaporation must have been greater in those not covered, and partly covered dishes, than in the covered dishes, and since some of these gave normal, it does not seem probable that enough evaporation takes place in covered Syracuse dishes to cause abnormal development (see below). The large number of eggs kept in the finger bowl gave nearly all normals.

Another experiment was made in the same way (Aug. 21, 1944). Cross-fertilized in finger bowls, the eggs in the two-cell stage were distributed in eight Stender dishes. Five lots gave nearly all normals, but one gave all abnormalities, and two gave more abnormalities than normals. In the large number of eggs left over in the two finger bowls, one gave 100 per cent normals and the other 99.9 per cent normals. Obviously the conditions in three of the Stender dishes were unfavorable.

#### *Effect of Evaporation of the Sea Water on Development*

One of the possible influences that cause abnormal development might be evaporation of the sea water from the covered Syracuse dishes. To test this, 10

cc. of sea water was evaporated in the dishes to a slight extent before the eggs, that had been fertilized in a large volume of water, were transferred to the concentrated sea water (Oct. 16, 1944). As a control, two autoclaved dishes that had been covered, but their volumes reduced only to  $9\frac{7}{10}$  and  $9\frac{5}{10}$  cc. gave 100 per cent normals. Another dish, where the water had evaporated to  $7\frac{9}{10}$  cc., gave all twisted and bent embryos. In another, evaporated to 7 cc., the eggs had remained in 2 cells after 24 hours. In general the water in a covered Syracuse dish is reduced during 24 hours by about  $\frac{3}{10}$  to  $\frac{5}{10}$  cc. As shown by these and other tests this amount of reduction does not interfere with normal development. Hence, the irregularities observed in the earlier experiments were not due to concentration of the sea water.

#### *Sterilization of Dishes by HCl*

Several tests in duplicate were made. In one (A by b, Sept. 3, 1944) the dishes had been sterilized with weak HCl and washed in distilled water and dried. There were eight that gave 95 to 100 normals, but two gave 100 per cent abnormals. Reciprocally, (B by a) there were six that gave normals (90 to 100 per cent) and four that gave mostly abnormals. The high frequency of dishes with abnormals may seem to be due to the acid, but if any was left after washing, it should have evaporated when the dishes were dried (24 hours). Many eggs were left in the finger bowls, and A by b and B by a each gave 100 per cent normals. Whatever the cause of occasional abnormal development, it appears that it was not removed from all the dishes by the HCl treatment. Either the HCl itself was not entirely removed by washing, or else something else was left behind after the treatment that was later the basis of contamination (infection) of the dishes. The result is in accord with earlier experiments of the same sort (*Biol. Bull.*, 80, 1941, pages 348-349).

#### *Sterilization of Dishes by Heat*

The records of the preceding experiments show almost without exception, one or more similarly treated dishes with abnormal embryos, while the rest contain only, or largely, normals. Such exceptions occur even when the eggs have been transferred to the smaller dishes at the two- or four-cell stage. The result suggests contamination or infection of some lots. Therefore, a new set of 10 duplicated lots and 10 reciprocals were tested in dishes that had been autoclaved ( $120^{\circ}$  C.). The eggs were collected from the oviduct in such a dish and transferred to a large flask (also autoclaved) and there cross-fertilized. At the two-cell stage, 50 to 100 eggs were transferred to five flasks and five Stenders each, 20 in all. After 24 hours, A by b gave practically all normals, i.e. 85 to 100 per cent. The reciprocals all gave 100 per cent, except one lot that had 50 per cent somewhat bent tadpoles and 50 per cent normals. The eggs left over in the large flasks (A by b) gave 99 per cent normals and one per cent bent, about two thousand in all. In the reciprocal, there were about one thousand normal tadpoles.

Another experiment was made the next day with Cionas brought to Pasadena; 20 dishes were prepared as above (autoclaved). The eggs were kept in Syracuse dishes. They gave 99.9 per cent normals. The many eggs (about two thousand) left in a large beaker (sterilized) also gave 99.9 per cent normals.

A third experiment of the same kind in which the dishes had been boiled for a short time instead of autoclaved, gave nearly the same results. Most of the dishes, A by b, (Syracuse) gave from 95 to 100 per cent normals; only one had 75 per cent normals and 25 per cent abnormals. The reciprocals (B by a) gave five 90 to 98 per cent normals; four, 80 per cent, and one, 75 per cent normals. One (A by b) of the large finger bowls (80 cc.) gave 99.5 per cent normals and 0.5 per cent bent; the other gave only 5 normals, 1 bent and 94 abnormals. It is evident that this finger bowl (B by a) was contaminated, but the eggs that were removed from it at the two-cell stage and transferred to the ten smaller autoclaved dishes with 10 cc. fresh sea water gave 80 to 95 per cent normals.

These sixty tests are very convincing that the cause of abnormal development is due to some contaminating agent present in the dishes, or that develops there (bacterial action). Washing the dishes in tap water and even rinsing them in distilled water does not remove the source of the contamination, while autoclaving the dishes is effective. It should be recalled that in most of the earlier experiments the embryos after 24 hours were killed in weak formalin and the dishes allowed to stand for several hours or days, before washing them again in tap water. They were then dried for a day or longer. Whether the formalin combined with some organic matter in the dishes, or whether the organic matter alone is responsible for the contamination is not shown by these experiments.

Another test was made (September 30–October 1, 1944) with fresh *Ciona* at Corona del Mar. The eggs were brought to Pasadena in autoclaved flasks and Stenders. Eggs had been fertilized in large finger bowls (80 cc.), and transferred in an early cleavage stage to 5 flasks and 5 stenders, 20 in all. Only normal tadpoles developed. Again, a similar experiment was carried out at Pasadena (Oct. 1–2), but the eggs were transferred to 20 Syracuse dishes that had not been autoclaved since the last time they were used. They gave 99 per cent normals in all, but 5 dishes (out of 20) gave 95 per cent. These two tests corroborated the conclusion that in clean dishes nearly 100 per cent are normal tadpoles. The very small percentage of abnormals may be due to polyspermy, or to other defects in the cleavage.

A different test was carried out as follows (Oct. 1, 1944). A set of 20 dishes was made up as above, using Syracuse dishes that had been washed, but not autoclaved. There were 14 that gave 95 per cent to 100 per cent normals; 4 that gave only abnormals and one that gave 80 per cent normals. The 4 that gave abnormals were carried one step further. The water was drawn off and put into an autoclaved Stender. To the original Syracuse dishes, 10 cc. of fresh sea water was added. Eggs of another individual, in the two cell stage were added to each. After 22 hours all of the Stenders had only abnormal embryos. Evidently the water had been fouled in some way. The six original dishes with fresh water gave 95 per cent normals, 5 per cent abnormals; 50 per cent normals; 100 per cent abnormals; 100 per cent normals; 95 per cent normals; and 80 per cent normals. These eggs did much better in the old dishes with fresh sea water than in the old sea water in a fresh dish.

As a control test, six of the original (Syracuse) dishes that had given only normals were treated in the same way. The six original dishes with fresh sea water (10 cc.) gave all normals. The old sea water from 4 dishes transferred

to Stenders gave in one dish normals, another gave abnormals, and another 50 normals and 50 abnormals. More normals resulted than when the original dishes that gave only abnormals were tested. These results are consistent with the assumption that abnormal development is due to toxic material that develops in some of the dishes. It does not seem to be due to the eggs themselves, but to some foreign material present in some of the dishes. Ordinary washing does not remove the material, but sterilization of the dishes does remove it. If the toxic material is due to bacteria left in the dishes, the protein or other substance on which the bacteria grow may be due either to some substance left in the dishes, or to organic material that goes over with the eggs or the sperm.

### SUMMARY

Eggs of *Ciona* that develop in covered Syracuse dishes produce as a rule normal tadpoles in the course of 24 hours, but occasionally only abnormal embryos develop, or both normal and abnormal in the same dish.

Reciprocal crosses sometimes give very different proportions of normals and abnormals, but the relations are often very inconsistent. The results cannot be ascribed to the eggs alone or to the sperm or to their combinations. Even if an attempt is made to refer the outcome of the reciprocal crosses to the cytoplasm of the egg, that has developed under the influence of the diploid nucleus of the egg, still the differences cannot be satisfactorily accounted for.

Polyspermy can account for only a very small percentage of abnormal development, probably not more than half of one per cent.

Delay in fertilizing the eggs does not cause abnormal development. Delay in using the sperm suspension does not cause abnormals.

When the eggs of one individual are fertilized by the same amount of sperm suspension of six other individuals, the outcome may be normals in each dish or normals and abnormals, or occasionally, all are abnormals. When the sperm of one individual is used to fertilize the eggs of six others, the same kind of results may happen.

In order to make the samples of eggs as nearly alike as possible, they were first fertilized in a large volume of water, and, when in the two-cell stage, a few were transferred to several small Syracuse dishes. The same kind of irregularities appeared, which cannot be due to chance selection of different eggs, for the original eggs left in the large amount of water generally gave more than 95 per cent normals.

Therefore, several kinds of experiments were made to find out whether differences in the small dishes will account for the occasional, but persistent, appearance of abnormals. (1) Dirt or impurities in the sea water from the tap was excluded. (2) Evaporation of the sea water in the covered Syracuse dishes was not enough to cause abnormal development. (3) Ordinary washing of the dishes, even rinsing them in distilled water did not remove the cause of abnormal development in some of the dishes. (4) But sterilizing the dishes in an autoclave removed the cause of most of the irregularities.

It follows that the cause of the exceptional cases of abnormals is due to some contamination that remains in the dishes after washing them in fresh tap water. Since the contamination does not affect the early development of eggs (cleavage),

but only later stages, it must be putrefactive in origin; and since it is removed by autoclaving the dishes, it is probably bacterial or some sort of organic contamination.

The eggs come to rest on the bottom of the dishes in a few minutes where they remain until the tadpole emerges, hence local differences may affect the development and account for those cases where both normal and abnormal development takes place in the same dish.

The discovery of the cause of the occasional abnormal development removes the possibility that it is due to genetic factors, hence is not concerned with the self-sterility of *Ciona*.

## REGULAR OCCURRENCE OF HETEROPLOIDY IN A GROUP OF PENTATOMIDAE (HEMIPTERA)

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The testis of all pentatomid Hemiptera is composed of lobes which are constant in number for any given species. In some species the size of the spermatocyte cells and sperms may vary in the different lobes, but in any one species there is a characteristic size for each lobe. In other words, if the first lobe in one specimen produces unusually large spermatocytes, the corresponding lobe in other specimens of that species will also show such large spermatocytes. This peculiarity was first discovered by Montgomery (1898), who described it in some detail in a later paper (1910). The phenomenon was called "polymegaly" by Bowen (1920, 1922a, 1922b) who in his exact analysis laid special stress on the processes of sperm formation. He found that certain cytoplasmic elements varied in amount proportionally to the size of the cells, but that the size of the chromosomes varied little or not at all in the different lobes. Normal sperms were formed from all lobes, and differences in the shape of the head arose only because there was greater elongation in some lobes.

The conditions that are basic to this constant variation are therefore subject to thoroughly regulated processes in any given species. They involve no mitotic irregularities and the occurrence of nondisjunction, multipolar spindles and failure of cytoplasmic division, which of course are found here as in other animals and plants, always seem to result from physiological accidents that are sporadic and in no way correlated with polymegaly.

It is therefore of some interest to record a series of cases among the Pentatomidae in which mitotic disturbances occur regularly in a certain lobe of the testis and affect all the cells of that lobe. Moreover such irregularity, although resulting in heteroploidy of an extremely large range, does not affect the basic processes of sperm formation. Hence spermatozoa are produced which vary greatly in volume, but which appear to be otherwise normal in general structure.

### MATERIAL AND METHODS

The pentatomids concerned are *Loxa flavicollis* Drury, of which nine specimens were collected in Panama (1940 and 1941) and Costa Rica (1944); *Loxa picticornis* Horvath, represented by six specimens from Panama (1940) and Costa Rica (1944); and *Loxa florida* Van Duz, collected in Florida, U. S. A., in 1909. The data on *Loxa florida* are contained in Bowen's papers (1922a and b) although the main points here at issue are not specifically noted by him. A fourth species, *Mayrinia variegata* Dist., is represented in my material by only a single male collected in Costa Rica in 1944. Though strikingly smaller than the uniformly large species of *Loxa*, it is nevertheless a close relative and was formerly classified in

that genus. Its affinity to the species of *Loxa* is now further attested by the fact that it shares with them the striking mitotic features that form the subject of this paper.

Both testes and ovaries were fixed in either Sanfelice or Allen's Bouin and stained with haematoxylin, gentian violet, or the Feulgen method. Although Bowen did not state it, it is more than likely that his material of *Loxa florida* for which he thanks Professor E. B. Wilson, was fixed in strong Flemming solution. In all of my own material the gonads were usually fixed within a short interval of capture, usually less than three hours and sometimes within a few minutes.

My thanks are due to a number of people who have aided me in collecting my material. In this respect I am especially obligated to Dr. T. J. Grant of the U. S. Department of Agriculture in Turrialba, Costa Rica.

#### STRUCTURE OF THE TESTIS

In all three species of *Loxa*, the testis is composed of seven lobes, which are arranged side by side in a single series. Following Bowen (1922b) these lobes are designated by consecutive numbers, the first being closest to the side toward which the vas deferens opens (Fig. 1). The lobes show a constant variation in diameter, but only one lobe is strikingly larger than the others. This is the fifth, which may have a diameter two or three times that of any other and which at the same time is characterized by spermatogonial and early spermatocyte cells that are distinctly smaller than the cells of other lobes. It is this lobe which regularly shows heteroploidy in the spermatocytes.

It is this lobe also which shows heteroploidy in *Mayrinia*. There however, the fifth is not the largest lobe, for it is exceeded in diameter by the sixth. In neither of the two *Mayrinia* testes at my disposal is a seventh lobe visible, and it may be that in this species the last two lobes have fused to form a single, larger one. Since there are some indications that my specimen is an old male, it is inadvisable to draw any general conclusions on this point. The main point however is fully established. In all four species it is the fifth lobe of the testis which always shows the special development here under discussion.

#### CYTOLOGY

In all three species of *Loxa*, the spermatocyte cells are smaller in the fifth than in the other lobes. But as Bowen (1922b) has pointed out in reference to polymegaly in general, this size difference rests in the volume of the cytoplasm rather than that of the chromosomes. In *Mayrinia*, such polymegaly is not as striking as in *Loxa*.

The spermatogonial divisions appear to be very much alike in all of the lobes (Fig. 2A and B). The fifth lobe shows the diploid set of 14 chromosomes differing in no perceptible way from that of the remaining lobes, and mitoses are orthodox. The unusual developments do not become evident until the prophase of the first spermatocyte. Since a detailed cytological analysis will be published elsewhere, only a brief outline will be given here.

The most obvious deviation from the normal course first occurs some time before diakinesis. When the cells of a given cyst reach this stage they begin to

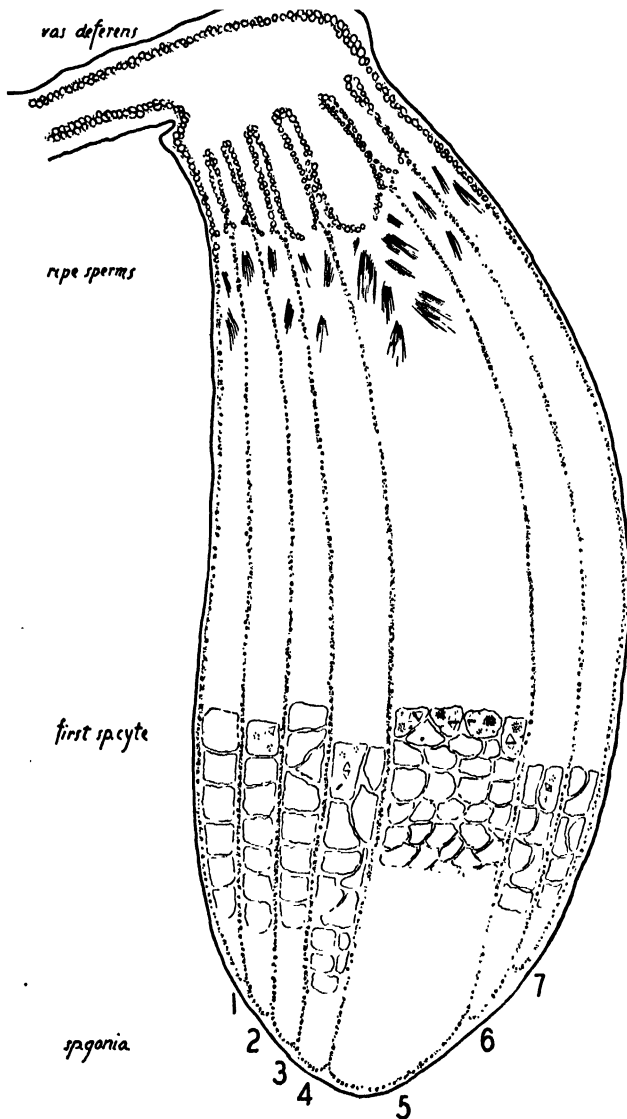


FIGURE 1. Section of the testis of *Loxa flavicollis*, showing the positions and comparative sizes of the seven lobes. Heteroploidy occurs regularly in the fifth lobe. Actual length of testis is 4 mm, and contents are only partially shown.

fuse with each other. The process is irregular in the sense that cellular aggregates resulting from the union of two or very few cells may lie side by side with much larger aggregates comprising many more cells. The nuclei at this time tend to become amoeboid and may divide by constriction, but if fusion occurs among them, it is not general.

Later during the prometaphase, cellular boundaries become very vague and the whole cyst may come to simulate a single, giant cell. It is at this time also that the nuclear walls disintegrate, as they do in the normal course of events. The chromosomes, condensing rapidly, then seem to lie scattered irregularly through the cytoplasm. However, immediately prior to the first appearance of spindles, the cytoplasm is segregated into discrete masses which do not seem

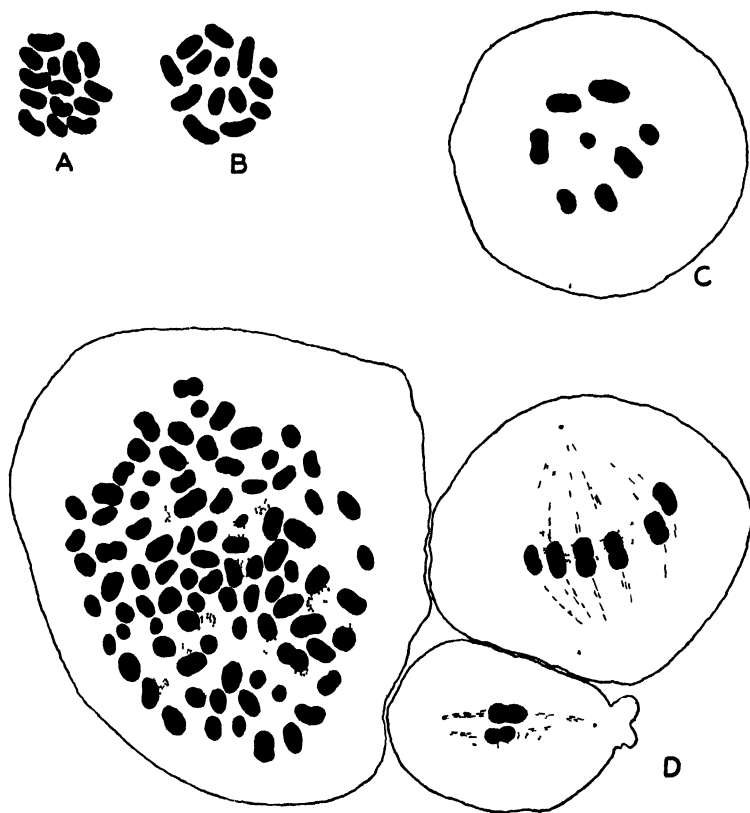


FIGURE 2. All from *Loxa flavicollis* (approx. 1,500  $\times$ )

A. Spermatogonial metaphase from normal lobe (14 chromosomes).

B. Spermatogonial metaphase from fifth lobe (14 chromosomes)

C. First spermatocyte metaphase from normal lobe (6 autosomal tetrads + X + Y).

D. First spermatocyte metaphases from fifth lobe (showing 3, 12, and upwards of 60 chromosomal bodies).

to correspond exactly to the original fusion aggregates. It is probable that this new step involves the activity of both chromosomes and centrioles in the formation of spindles, and the latter appear very soon thereafter. Simultaneously the chromosomes are arranged into numerous metaphase plates, the number of which corresponds to the rounded masses of cytoplasm just formed. Since the latter seem to have no reference to the original prophase spermatocyte cells, the different metaphases may be composed of as few as two (perhaps even one) and

as many as two hundred or more chromosomes (Fig. 2D). By the same token, the types of chromosomes included in such metaphase plates vary greatly. All in all, these configurations of chromosomes are the result of rather haphazard processes and it is probably only very rarely that they happen to represent the orthodox set of chromosomes (Fig. 2C) in the first spermatocyte (i.e. six autosomal tetrads + X + Y), especially since it seems doubtful whether normal pairing occurs in the fifth lobe.

Although the number of multipolar spindles is distinctly higher than in normal lobes, it is not as great as one might expect from the seemingly confused conditions that prevail just before spindle formation. Unlike the chromosomal movements in the preceding period, the maneuvers of the centrioles are evidently not completely

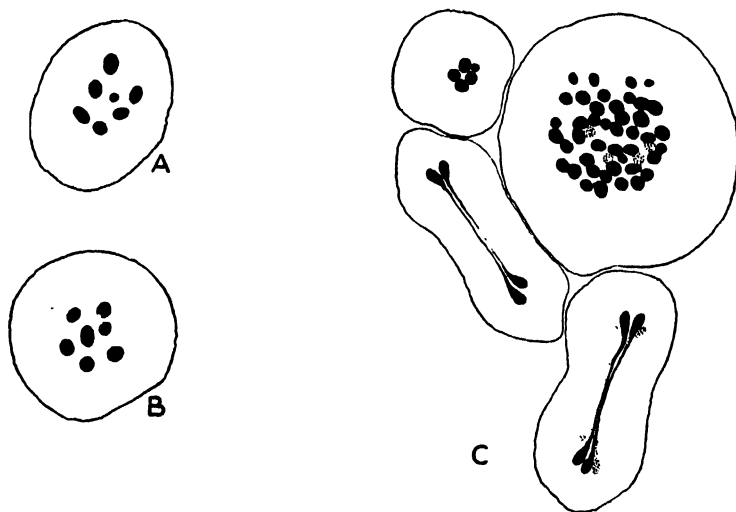


FIGURE 3. All from *Loxa flavicollis* (approx. 1,500  $\times$ ).

A. Chromosomes of telophase of second spermatocyte in normal lobe (6 autosomes + Y).

B. Chromosomes of telophase of second spermatocyte in normal lobe (6 autosomes + X).

C. Chromosomes of telophase of second spermatocyte in fifth lobe (showing 2, 4, 5, and upwards of 40 chromosomes at each pole).

at random and their final positions result in bipolar figures in the great majority of cases.

Despite the bewildering range in the composition of these metaphase plates, the actual division of the abnormal first spermatocytes occurs normally. The second spermatocytes therefore carry a correspondingly great variation in the numbers of chromosomes and they too divide successfully to give spermatid cells (Fig. 3).

The nuclei that are formed in the spermatids after the second spermatocyte division of course reflect the variation in the chromosome numbers that are encountered in the meiotic divisions. Hence they may greatly exceed in size the normal spermatid nucleus, and such large nuclei may lie side by side with the tiny nuclei derived from only one or two chromosomes (Fig. 4). However

except for this deviation from the normal size, the processes of sperm formation seem to parallel those observed in the normal lobes. The behavior of chondriosomes and Golgi material seems to be regular, and the transformations of the nucleus that culminate in the tenuous head of the ripe sperm are undergone just as in normal cells. The number of degenerating sperms is not great and certainly a huge number reach the final stages near the entrance to the vas deferens. Since cells with a normal complement of chromosomes must be very rare indeed, there is no escaping the fact that very many sperms carrying irregular combinations and numbers of chromosomes reach the final stages of development. Whether they then enter the egg and become functional has not been determined.

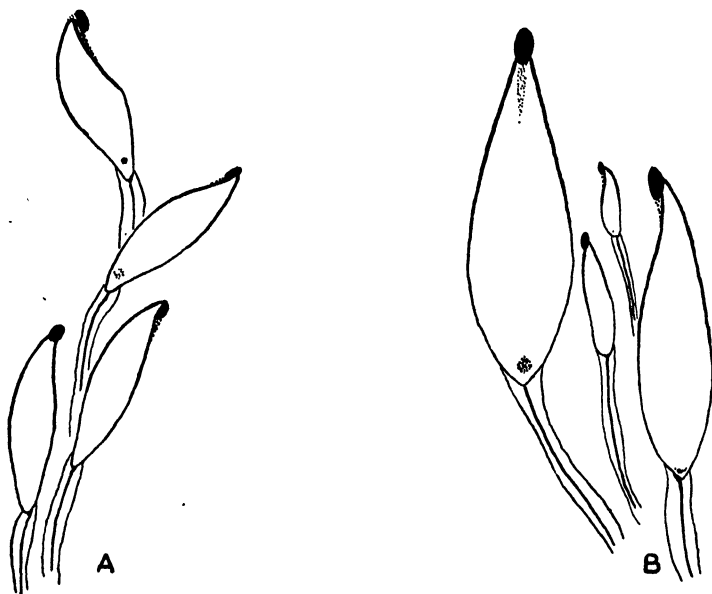


FIGURE 4. All from *Loxa flavicollis* (approx. 1,500 $\times$ ).

A. Sperms (only heads shown complete) at about Stage n. from normal lobe.

B. Sperms (only heads shown complete) at about Stage n. from fifth lobe, showing great variation in size.

The evidence that *Loxa florida* duplicates the conditions here described, is indirect but conclusive. In one of his papers, Bowen (1922a) discusses the occurrence of abnormally high numbers of chromosomes in that species, and ascribes them to a fusion of spermatocyte cells. He mentions that this takes place in the same lobe of both the *Loxa* testes available to him. Although not stating which lobe this is, Bowen characterizes it as carrying unusually small spermatogonial and early spermatocyte cells. In a somewhat later paper of that year (1922b) he mentions that in *Loxa florida* it is the fifth lobe that has cells which are distinctly smaller than those of the other lobes. There is thus no reason to doubt that he was dealing with conditions in *Loxa florida* that are essentially the same as those here described and which there occur also in the fifth lobe of the testis.

## EVOLUTIONARY ASPECTS

These then are the astonishing conditions that confront us in the two genera, *Loxa* and *Mayrinia*. In four different species, taxonomically distinct, and collected from Panama, Costa Rica, and Florida—a latitudinal range of at least 1,700 km. (or about 1,050 miles)—one of the lobes of every testis undergoes a very special type of abnormal development. This abnormality results in a great number of unusual distributions of the chromosomes, but is in its basic nature identical in all four species. For in all of them it is always the fifth lobe of the testis that is affected; in all of them the spermatogonial divisions of this lobe seem normal and cytological indications of abnormality do not appear until the preparatory phases of the meiotic period; and in all four species the odd assortments of chromosomes that result partake nevertheless in a formation of sperms which are normal in appearance except for size.

Whatever the factors may be that bring about this constant and regularly occurring abnormality, its evolutionary aspects pose some interesting questions. It is reasonable to assume that the conditions underlying it are of long standing in the history of these species and that in fact they were probably present in the parent form from which they arose. Since all of my sixteen specimens carry the orthodox complement of 14 chromosomes, the indications are that the great majority of the sperms with abnormal chromosomes numbers are either nonfunctional or produce nonviable zygotes. The existence of so wasteful a development would seem to be an incongruity which might well be expected to meet with short shrift in the processes of natural selection. Nevertheless it has persisted, either because it involves some benefit to the species that is not immediately obvious or else because the elaboration of useless sperms, even in such huge numbers, entails only an imperceptible drain on the evolutionary welfare of the animal since it is amply compensated by the production of sperms from the normal lobes of the testis.

Be that as it may, the constant production of gametes with irregular chromosome numbers should greatly enhance the chances for producing zygotes with other than the usual complement of 14 chromosomes. Granted that most of the abnormal sperms will not produce viable zygotes, it is likely that some of the many unusual combinations do at times develop. Certainly the flow of new and heteroploid forms from such a species should be very much greater than in the case of species in which the opportunities for such varietal changes are restricted to the comparatively rare mitotic accidents that occur in the more orthodox processes of germ cell formation. It may be that a basic conservatism that may exist among the Pentatomidae is not favorable to such evolutionary adventures, but the number of specimens at my disposal is surely too small to allow of any final conclusions on this point. Certain it is that a further examination of the 33 so far described species that belong to the three, taxonomically closely knit genera *Loxa*, *Mayrinia*, and *Chlorocoris* may well throw light on these questions (all except *Loxa florida* are exclusively neotropical, but their range extends from Florida to Argentina; see Van Duzee, 1909 and Horvath, 1925).

But if, as already suggested, the conservatism of these Hemiptera does not allow a utilization of such evolutionary opportunities, the case nevertheless presents a mechanism which in some other groups might well be conducive to rapid and great strides in the elaboration of new species. The ready viability of forms with unusual

chromosome combinations such as characterizes for instance genera like *Datura* and *Crepis* would suggest that other forms might well take advantage of such a range of possibilities. At any rate, the case indicates in what unexpected ways a group of organisms may suddenly become very active in the evolutionary sense.

#### DIFFERENTIATION OF SPERMS

The case reflects in a rather striking, albeit not entirely novel way, on the problem of differentiation. It is clear that the complex processes which bring about the formation of the highly specialized sperm—including the special transformations of Golgi and chondriosome material as well as the change of the spherical spermatid nucleus to the extremely tenuous, chromatic sperm head—all occur regardless of whether only one or two or many dozens of chromosomes are present in the cell. It therefore would seem safe to conclude that the immediate control over these developments is vested in cytoplasmic elements and that, if nuclear genes are primarily responsible they must exert their influence on the cytoplasm at least two mitotic cycles earlier (in the spermatocyte prophase when cell fusion first occurs). A demonstration to the same effect is contained in the work of Dobzhansky and Sturtevant (1931) where it is shown that sperms of *Drosophila* which have lost various sections of the chromosomes through translocation during the meiotic prophase, nevertheless become functional.

#### SUMMARY

1. The fifth lobe in the testes of four species of *Loxa* and *Mayrinia* regularly produces sperms carrying heteroploid complements of chromosomes.
2. This condition must be of long evolutionary standing and may be of significance in the formation of new forms.

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ASEXUAL REPRODUCTION IN THE COLONIAL TUNICATE,  
BOTRYLLUS SCHLOSSERI (PALLAS) SAVIGNY, WITH  
SPECIAL REFERENCE TO THE DEVELOPMENTAL  
HISTORY OF INTERSIPHONAL BANDS OF  
PIGMENT CELLS

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INTRODUCTION

*Botryllus schlosseri* (Pallas) Savigny<sup>2</sup> is a colonial tunicate readily obtained in the Eel Pond at Woods Hole, Massachusetts. In a well developed colony the individual blastozooids (or ascidiozooids) are grouped into one or more systems; each system consists of 2 to 23 blastozooids radiating outwards from a central common cloaca with the separate oral siphons distributed at the periphery. The most striking feature of many *Botryllus* colonies is the localization of special light-reflecting pigment cells between the oral and atrial siphons of each blastozooid. Because of the association of individual blastozooids into systems within each colony the total aggregation of such reflecting cells within any one system forms an attractive star-shaped pattern (Pl. 1, Fig. 3) clearly revealing the spatial distribution of the blastozooids involved. Such patterns will be referred to as intersiphonal patterns of pigment cells (intersiphonal patterns for short), and each arm of the pattern will be called an intersiphonal band of pigment cells (intersiphonal band for short).

Such intersiphonal bands have been described more or less incidentally by various taxonomists; however, only two of these seemed to realize that the bands are actually aggregations of pigment cells in specific regions. Both of these men realized that these intersiphonal bands are not constant in their appearance. Pizon (1899a) merely states that they undergo changes with time without specifying the nature of these changes. Bancroft (1903) states that in young zooids there are no intersiphonal bands at all and that three to four days elapse before they reach their complete formation. Such brief observations constitute the only information available in the literature concerning the developmental history of intersiphonal bands

<sup>1</sup> It is a pleasure to acknowledge the excellent assistance of Miss Juanita Senyard during the accumulation of the data upon which this paper is based.

<sup>2</sup> Many species and varieties of *Botryllus* have been described (Giard, 1872; Herdman, 1891; Hartmeyer, 1909-1911; Alder and Hancock, 1912). However, these were distinguished largely on the basis of color differences. Pizon (1899a) early recognized the need to revise the classification of *Botryllus* since he realized that considerable color variation may occur even within the same colony, and Bancroft (1903) even went so far as to state (p. 161), "in *Botryllus*, as it occurs in Europe and the Atlantic Coast of North America, color characters cannot be used for separating species; . . . therefore, since none of the described species have been based upon morphological characters, there is no valid reason for recognizing more than the single species, *B. schlosseri* (Pallas, 1766, pp. 355-356) Savigny (1816)." This view is acceptable to Van Name (1910) and to Herdman (1925).

of pigment cells. This lack of information is rather surprising since the bands are so very striking when fully formed (Pl. I, Fig. 5) and in view of the fact that so much has been written concerning other features of *Botryllus*.

One of the most interesting features of these intersiphonal bands has received no attention at all, viz., that they are not permanent additions to the pigment pattern of the colony; instead, as soon as they have formed, they are completely destroyed, and this destructive phase marks the most radical change in the appearance of the colony (Pl. II, Fig. 8). This destructive phase is inevitable since, as is well known (Berrill, 1941c), the parent zooids degenerate each time a new generation of zooids arises by asexual reproduction, and these intersiphonal bands are properties of the individual zooids, not of the colony as a whole. The fact that they give to the colony a characteristic intersiphonal pattern or group of patterns is only secondary.

This paper has a twofold purpose: (1) To describe the visible changes in the intersiphonal bands of pigment cells during the establishment of the colony from the larva. By observing the development of a number of colonies it is possible to correlate rather accurately the steps involved in the formation and destruction of the intersiphonal bands with other known steps in asexual reproduction. (2) To describe the variation in the intersiphonal patterns when different colonies are compared, and to consider possible factors involved in such variations.

#### MATERIALS AND METHODS

The eggs of *Botryllus* undergo fertilization and development up to a tadpole stage within the blastozooids. Each day some of these tadpoles escape from the cloaca. In order to obtain them adult colonies were collected from the Eel Pond in the morning and in the laboratory they were distributed in ten inch finger bowls filled with sea water. As the larvae escape from the parent zooids they swim toward the light and toward the surface (see Grave and Woodbridge, 1924, for time of liberation of larvae and for reactions of the larvae to light and gravity) and they can be collected easily with a pipette; three of them were placed into a large drop of sea water in the center of a syracuse watch glass and were left to attach and metamorphose into the oozoid. The watch glasses were stacked to prevent evaporation. Many of these tadpoles metamorphose without attaching (Grave, 1937) or exhibit certain indications of abnormal metamorphosis (Zhinkin, 1939), but these were discarded. Usually at least one tadpole attached in each watch glass; in some instances two or even all three larvae attached, and if they were sufficiently isolated from one another, they were allowed to continue their development. After attachment had occurred the watch glasses were immersed in large aquaria through which sea water was circulating. The watch glasses were inverted so debris would not settle out too thickly and obscure the development of the colonies, and they were held in this position by wooden racks. Each day these watch glasses were removed from the aquaria and the appearance of the colonies developing within them was sketched under the dissecting microscope with reflected light for illumination, since by such illumination only the distribution of the special light reflecting pigment cells is revealed and all other pigmentation of the zooids is automatically eliminated from observation (see Plates I and II). The exact time at which each sketch was made was recorded. Whenever a colony was

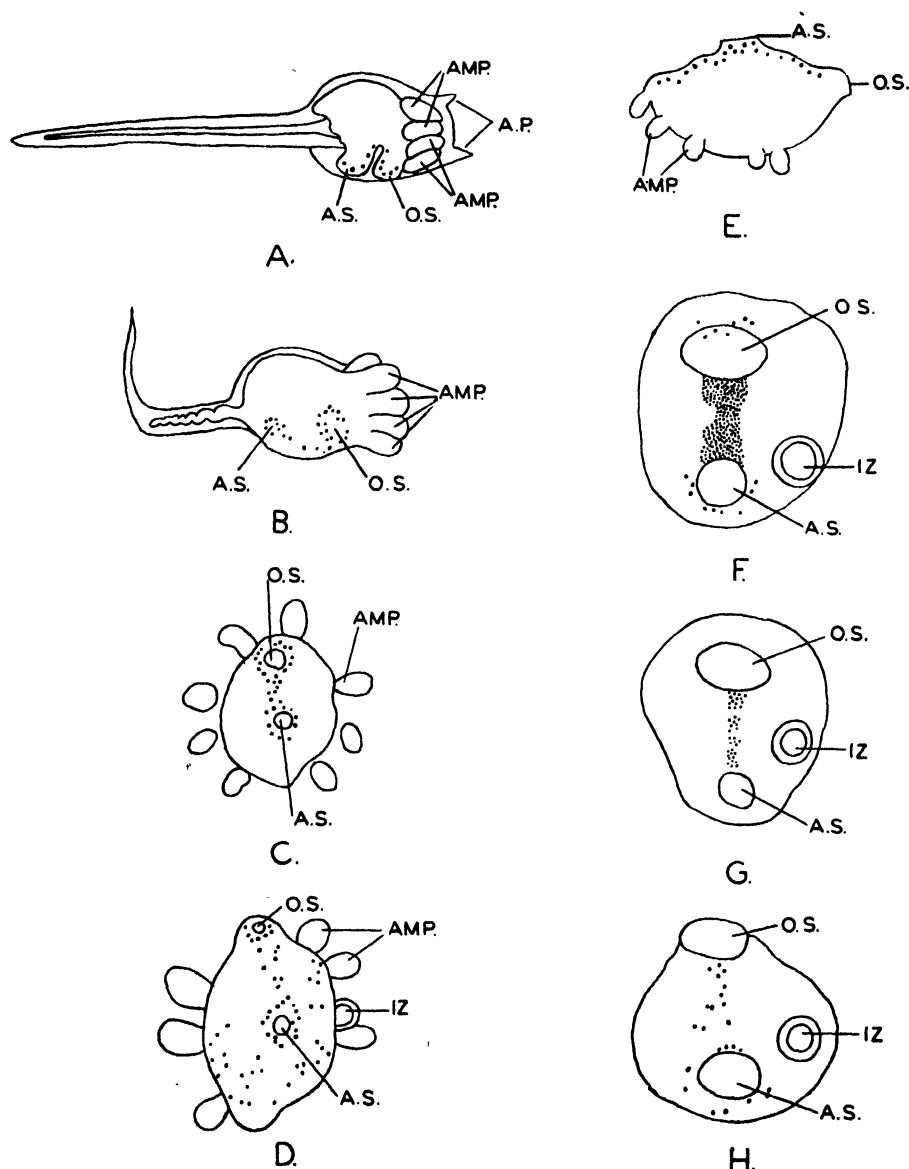


FIGURE 1. The development of intersiphonal bands of pigment cells in the oozoid. Each dot represents one pigment cell. a. Botryllus tadpole 20 to 30 minutes after escape from parent zooid. b. Tadpole in which the tail is undergoing absorption; one hour after escape from parent zooid. c. Oozoid eight hours after escape from parent zooid. d. Another oozoid 11 hours after escape from parent zooid. e. An optical section of an oozoid eight hours after escape from parent zooid. f. Oozoid No. 8c; age two days; example of a *strong* intersiphonal band of pigment cells. g. Oozoid No. 10; age two days; example of a *medium* intersiphonal band. h. Oozoid No. 18c; age two days; example of a *weak* intersiphonal band. Abbreviations: a.p. = adhesive papillae; amp. = ampullae; a.s. = atrial siphons; o.s. = oral siphons; IZ = first generation blastozooids.

in a crucial stage of transformation it could be watched for considerable periods of time without injury. However, because of the large number of colonies studied, continuous observations on any one colony for long periods of time were not possible, although such a procedure would have yielded useful supplementary information. All sketches made for each colony were then mounted from left to right in a horizontal row on large sheets of paper in the order in which the sketches were made, and they were so arranged on these sheets that sketches of all colonies made on any one day lie in vertical columns. Consequently the changes in the appearance of any one colony throughout the period studied can be ascertained quickly by running the eye horizontally across the sheet; and similarly the appearance of all the colonies on a given day is readily compared by running the eye vertically down the sheet. Sixty-one colonies were started from isolated larvae on July 31, 1942. These were first sketched on August 2, next on August 4, and then at daily intervals until August 19 if they still survived. Due to the increased complexity of the colonies by this time it was impossible to continue daily observations on all of them. Ten colonies were therefore selected and were sketched at daily intervals until September 4. Twenty-nine other colonies were allowed to develop until August 29 when sketches were made of them; however, for these colonies the daily changes between August 19 and August 29 are unknown. In the case of the remaining 22 colonies no sketches were made after August 19 for various reasons. Consequently, since only incomplete records are available for these 22 colonies, they will not be included in the general observations given below. In order to present these observations the daily changes in the appearance of one colony (No. 38) selected as an example will be described in detail (Fig. 2); the observations on the other colonies can then be presented briefly (Table I) by merely emphasizing their similarities and differences when compared with this example. All observations have been made exclusively on living material.

## OBSERVATIONS

### *Formation and Variation of Intersiphonal Bands in Oozoids*

Since the main series of colonies started on July 31 was first examined two days later it was necessary to start other colonies (on August 31 and September 3) in order to study the establishment of the first intersiphonal band during metamorphosis of the tadpole into the oozoid. These colonies served only for these early observations on oozoid development and were then discarded. Although the tadpole is rather opaque a few reflecting pigment cells can sometimes be seen in a newly emerged larva (Fig. 1a). By transmitted light these cells appear yellowish and by reflected light they are just barely visible. They are quite definitely localized in the dorsal surface and already outline the siphonal areas. Within an hour after the escape of the larva from the parent these pigment cells have darkened

FIGURE 2. The developmental history of intersiphonal bands of pigment cells in colony No. 38. Each dot represents a pigment cell. See text for age of colony at stages illustrated and for description of the progressive changes. The various stages are not drawn accurately to scale. Each degenerating zooid is indicated by cross-hatching. Abbreviations: c.c. = common cloaca; o.s. = oral siphons; s. = space free from pigment cells; 1Z to 7Z = first to seventh generation blastozooids.

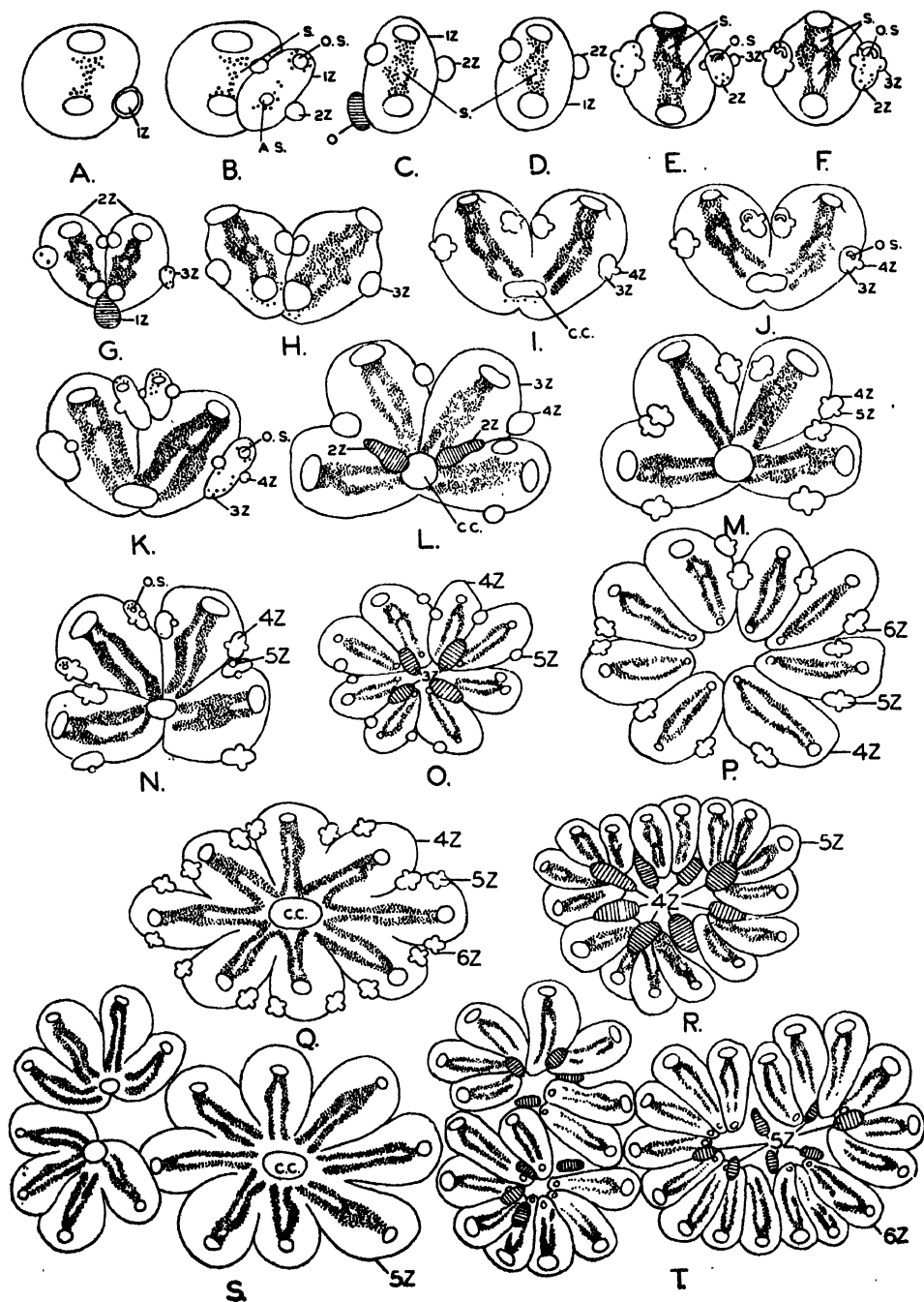


FIGURE 2

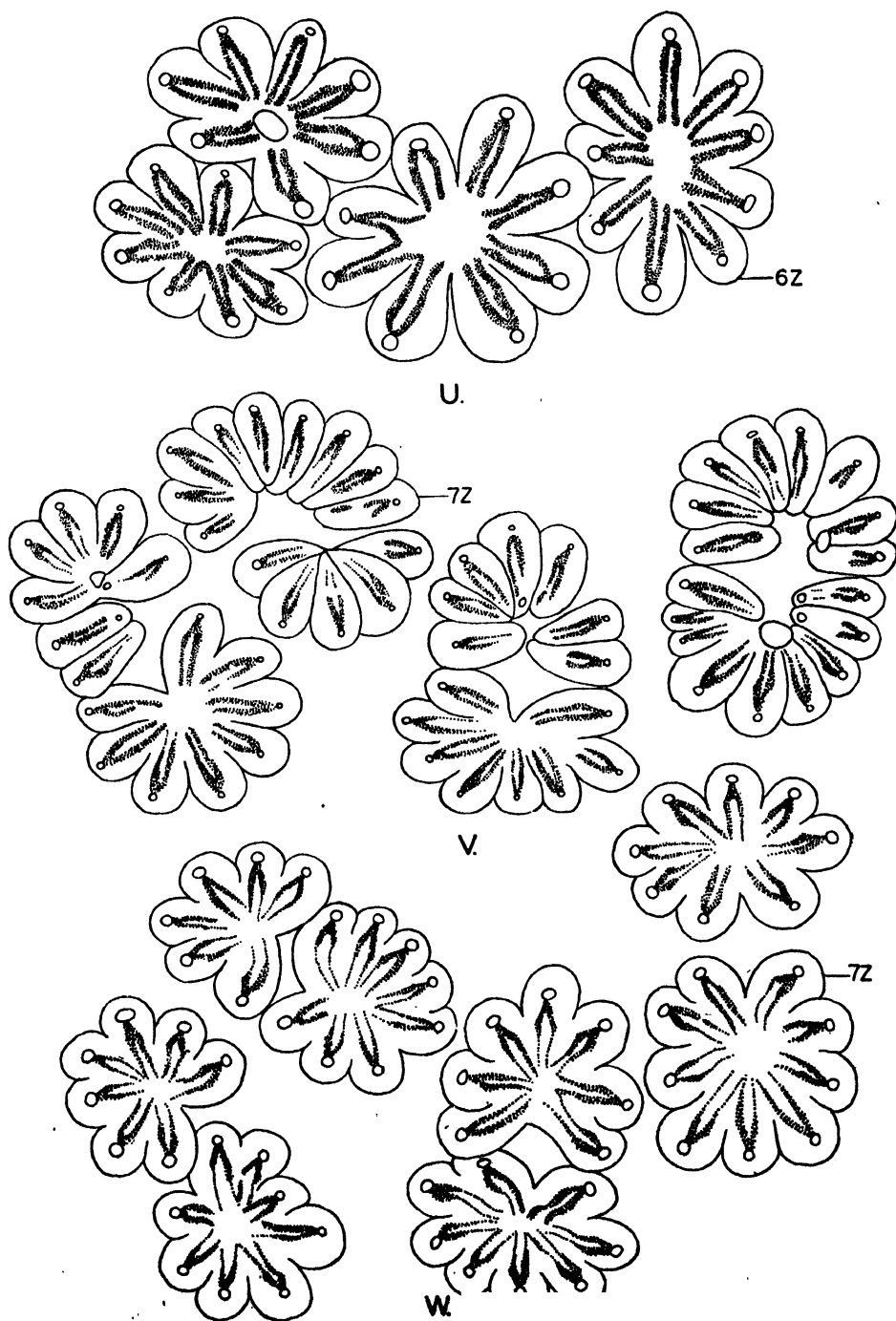


FIGURE 2—Continued

TABLE I

Summary of the relationship between changes in the intersiphonal bands of pigment cells and the major changes in asexual reproduction in all colonies studied. Each number indicates the percentage of colonies undergoing the change indicated. The number in parentheses following each percentage indicates the generation of zooids involved (0 = oozoid, 1-7 = first 7 generations of blastozooids). The percentages indicated by the asterisk may be too small since parent zooids obscure the buds in advanced stages of colony formation.

| Age in days | Zooids degenerating | Pigment cells circulating | Inter-siphonal bands single | Inter-siphonal bands double | Buds enlarging | Oral siphons present | Atrial siphons present | Common cloacas formed | Non-growing buds present |
|-------------|---------------------|---------------------------|-----------------------------|-----------------------------|----------------|----------------------|------------------------|-----------------------|--------------------------|
| 5           | 59.0(0)             | 30.7                      | 33.3(1)                     | 0.0(1)                      | 59.0(2)        | 0.0(2)               | 0.0(2)                 | 0.0(2)                | 0.0(3)                   |
| 6           | 41.0(0)             | 33.3                      | 61.5(1)                     | 5.1(1)                      | 94.9(2)        | 0.0(2)               | 0.0(2)                 | 0.0(2)                | 17.9(3)                  |
| 7           | 2.6(0)              | 7.7                       | 5.1(1)                      | 56.4(1)                     | 100.0(2)       | 15.4(2)              | 0.0(2)                 | 0.0(2)                | 94.9(3)                  |
| 8           | 0.0(0)              | 7.7                       | 7.7(1)                      | 56.4(1)                     | 100.0(2)       | 51.3(2)              | 28.2(2)                | 0.0(2)                | 100.0(3)                 |
| 9           | 35.9(1)             | 35.9                      | 17.9(2)                     | 2.6(2)                      | 15.4(3)        | 94.9(2)              | 94.9(2)                | 0.0(2)                | 0.0(4)                   |
| 10          | 41.0(1)             | 23.0                      | 28.2(2)                     | 25.6(2)                     | 82.1(3)        | 100.0(2)             | 100.0(2)               | 23.0(2)               | 7.7(4)                   |
| 11          | 7.7(1)              | 2.6                       | 20.5(2)                     | 43.6(2)                     | 97.4(3)        | 100.0(2)             | 100.0(2)               | 66.7(2)               | 74.1(4)                  |
| 12          | 2.6(1)              | 12.8                      | 7.7(2)                      | 56.4(2)                     | 100.0(3)       | 23.0(3)              | 0.0(3)                 | 69.2(2)               | 97.4(4)                  |
| 13          | 23.0(2)             | 12.8                      | 2.6(2)                      | 51.3(2)                     | 5.1(4)         | 41.0(3)              | 5.1(3)                 | 0.0(3)                | 97.4(4)                  |
| 14          | 48.7(2)             | 38.5                      | 30.7(3)                     | 2.6(3)                      | 48.7(4)        | 41.0(3)              | 41.0(3)                | 7.7(3)                | 2.6(5)                   |
| 15          | 23.0(2)             | 7.7                       | 17.9(3)                     | 28.2(3)                     | 84.6(4)        | 84.6(3)              | 84.6(3)                | 51.3(3)               | 20.5(5)                  |
| 16          | 5.1(2)              | 2.6                       | 7.7(3)                      | 48.7(3)                     | 100.0(4)       | 2.6(4)               | 0.0(4)                 | 66.7(3)               | 76.9(5)                  |
| 17          | 5.1(3)              | 2.6                       | 0.0(3)                      | 56.4(3)                     | 100.0(4)       | 25.6(4)              | 0.0(4)                 | 0.0(4)                | 89.7(5)                  |
| 18          | 23.0(3)             | 7.7                       | 5.1(4)                      | 5.1(4)                      | 20.5(5)        | 56.4(4)              | 15.4(4)                | 5.1(4)                | 2.6(6)                   |
| 19          | 23.0(3)             | 17.9                      | 23.0(4)                     | 17.9(4)                     | 87.2(5)        | 94.9(4)              | 76.9(4)                | 38.5(4)               | 10.3(6)                  |
| 20          | 0.0(3)              | 0.0                       | 10.0(4)                     | 80.0(4)                     | 100.0(5)       | 100.0(4)             | 90.0(4)                | 90.0(4)               | 20.6(6)                  |
| 21          | 10.0(3)             | 10.0                      | 10.0(4)                     | 80.0(4)                     | 100.0(5)       | 100.0(4)             | 90.0(4)                | 90.0(4)               | 80.0(6)                  |
| 22          | 10.0(4)             | 10.0                      | 0.0(5)                      | 10.0(5)                     | 90.0(5)        | 10.0(5)              | 0.0(5)                 | 0.0(5)                | 100.0(6)                 |
| 23          | 40.0(4)             | 40.0                      | 10.0(5)                     | 50.0(5)                     | 60.0(6)        | 60.0(5)              | 60.0(5)                | 50.0(5)               | 0.0(7)                   |
| 24          | 20.0(4)             | 20.0                      | 10.0(5)                     | 80.0(5)                     | 90.0(6)        | 90.0(5)              | 90.0(5)                | 80.0(5)               | 0.0(7)                   |
| 25          | 0.0(4)              | 0.0                       | 0.0(5)                      | 90.0(5)                     | 100.0(6)       | 90.0(5)              | 90.0(5)                | 90.0(5)               | 0.0(7)                   |
| 26          | 10.0(4)             | 20.0                      | 0.0(5)                      | 100.0(5)                    | 100.0(6)       | 100.0(5)             | 100.0(5)               | 90.0(5)               | 30.0(7)                  |
| 27          | 10.0(5)             | 10.0                      | 0.0(6)                      | 10.0(6)                     | 100.0(6)       | 10.0(6)              | 10.0(6)                | 0.0(6)                | *30.0(7)                 |
| 28          | 30.0(5)             | 10.0                      | 0.0(6)                      | 40.0(6)                     | 100.0(6)       | 40.0(6)              | 20.0(6)                | 20.0(6)               | *40.0(7)                 |
| 29          | 10.3(5)             | 12.8                      | 2.6(6)                      | 35.9(6)                     | 100.0(6)       | 61.5(6)              | 61.5(6)                | 51.3(6)               | 100.0(7)                 |
| 30          | 0.0(5)              | 0.0                       | 0.0(6)                      | 100.0(6)                    | 100.0(7)       | 100.0(6)             | 100.0(6)               | 100.0(6)              | 0.0(8)                   |
| 31          | 0.0(6)              | 0.0                       | 0.0(6)                      | 100.0(6)                    | 100.0(7)       | 100.0(6)             | 100.0(6)               | 100.0(6)              | *0.0(8)                  |
| 32          | 0.0(6)              | 0.0                       | 0.0(7)                      | 10.0(7)                     | 100.0(7)       | 10.0(7)              | 10.0(7)                | 10.0(7)               | *0.0(8)                  |
| 33          | 20.0(6)             | 0.0                       | 0.0(7)                      | 40.0(7)                     | 100.0(7)       | 40.0(7)              | 40.0(7)                | 10.0(7)               | *0.0(8)                  |
| 34          | 20.0(6)             | 20.0                      | 0.0(7)                      | 80.0(7)                     | 100.0(7)       | 70.0(7)              | 60.0(7)                | 60.0(7)               | *0.0(8)                  |
| 35          | 0.0(6)              | 0.0                       | 0.0(7)                      | 100.0(7)                    | 100.0(7)       | 100.0(7)             | 100.0(7)               | 100.0(7)              | *0.0(8)                  |

until they appear brownish by transmitted light and are more readily visible by reflected light; they now outline the siphonal region rather sharply (Fig. 1b). In some instances one or two of these reflecting cells are also visible in the ampullae (amp., Figs. 1a-1e). No great increase in the number of reflecting cells seems to take place during metamorphosis, at least not during the first day. Seven to eight hours after emergence of the larva the siphons are readily visible and typically the reflecting cells are restricted sharply to the areas immediately surrounding and between the siphons (Fig. 1c); however, in a few instances they are more widely distributed to either side of the dorsal midline as well (Fig. 1d). That these cells are very definitely restricted to the dorsal surface of the oozoid can be seen in optical section (Fig. 1e).

When the oozoids of the colonies started July 31 were first examined on August 2 considerable variation was noted in the appearance of their intersiphonal bands; however, the intersiphonal band of any oozoid could be classified quite readily as one of three types, either: a. *strong*, in which case there is a broad, almost solid band of reflecting cells between the two siphons (Fig. 1f); b. *medium*, in which case there is a band of pigment cells between the two siphons, but it is usually rather narrow or irregular and consists of relatively few reflecting cells (Fig. 1g); c. *weak*, in which case there are only a few scattered reflecting cells near the dorsal midline (Fig. 1h) with no obvious arrangement into a band. Twenty-nine of the colonies studied arose from oozoids classified as possessing *strong* intersiphonal bands, 13 from oozoids with *medium* intersiphonal bands and 16 from those with *weak* bands. Three colonies had abnormal oozoid stages in which the intersiphonal bands could not be classified.

*Destruction of Intersiphonal Bands in Oozoids and Their Formation in First Generation Blastozoids*

Each oozoid reproduces asexually by budding to produce the first generation blastozoid (1Z).<sup>3</sup> This bud is a double walled structure located on the right side of the oozoid; its inner layer is an evagination of part of the atrial wall of the parent oozoid; its outer layer is an evagination of the overlying epidermis. The atrial portion of the bud consists of formative cells which give rise to all parts of the blastozoid except the epidermis which is derived directly from the epidermis of the parent. By the time the siphons of the first generation blastozoid are established the oozoid begins to degenerate; its intersiphonal band is destroyed and the oozoid itself almost completely disappears. Meanwhile an intersiphonal band is established in the first blastozoid.

<sup>3</sup> The details of asexual reproduction in *Botryllus* have been presented rather fully elsewhere (Della Valle, 1882; Hjort, 1892; Oka, 1892; Pizon, 1893; Berrill, 1941a, 1941b, 1941c). However, some indication of the process is necessary here and also at other points in order to understand the progressive changes in the intersiphonal patterns recorded in this paper.

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PLATE I

All figures are photographs of the dorsal surface of the colonies taken by reflected light. Magnification ca. 7 X.

FIGURE 3. Colony 30c. Age 33 days. 6Z zooids with well developed intersiphonal bands.

FIGURE 4. Colony 35. Age 28 days. 6Z zooids distributed in two systems. Intersiphonal bands only partially developed and therefore not completely double. 5Z zooids degenerated on the 27th day.

FIGURE 5. Colony 23. Age 29 days. 6Z zooids distributed in four systems. Intersiphonal bands well developed.

FIGURE 6. Colony 13C. Age 28 days. Zooids highly sensitive and contractile resulting in the contracted state and irregularity of the intersiphonal patterns. Reflecting pigment cells are just appearing in the circulatory system. This is the first stage of the destruction of intersiphonal patterns. On the 29th day these 5Z zooids undergo degeneration and are replaced by 6Z zooids.

FIGURE 7. Colony 41. Age 28 days. 5Z zooids with well developed double intersiphonal bands. Nine 6Z zooids are faintly visible between the 5Z zooids. Note that intersiphonal bands are already appearing on 6Z zooids (arrow) before 5Z zooids have contracted. This is an exceptional condition.

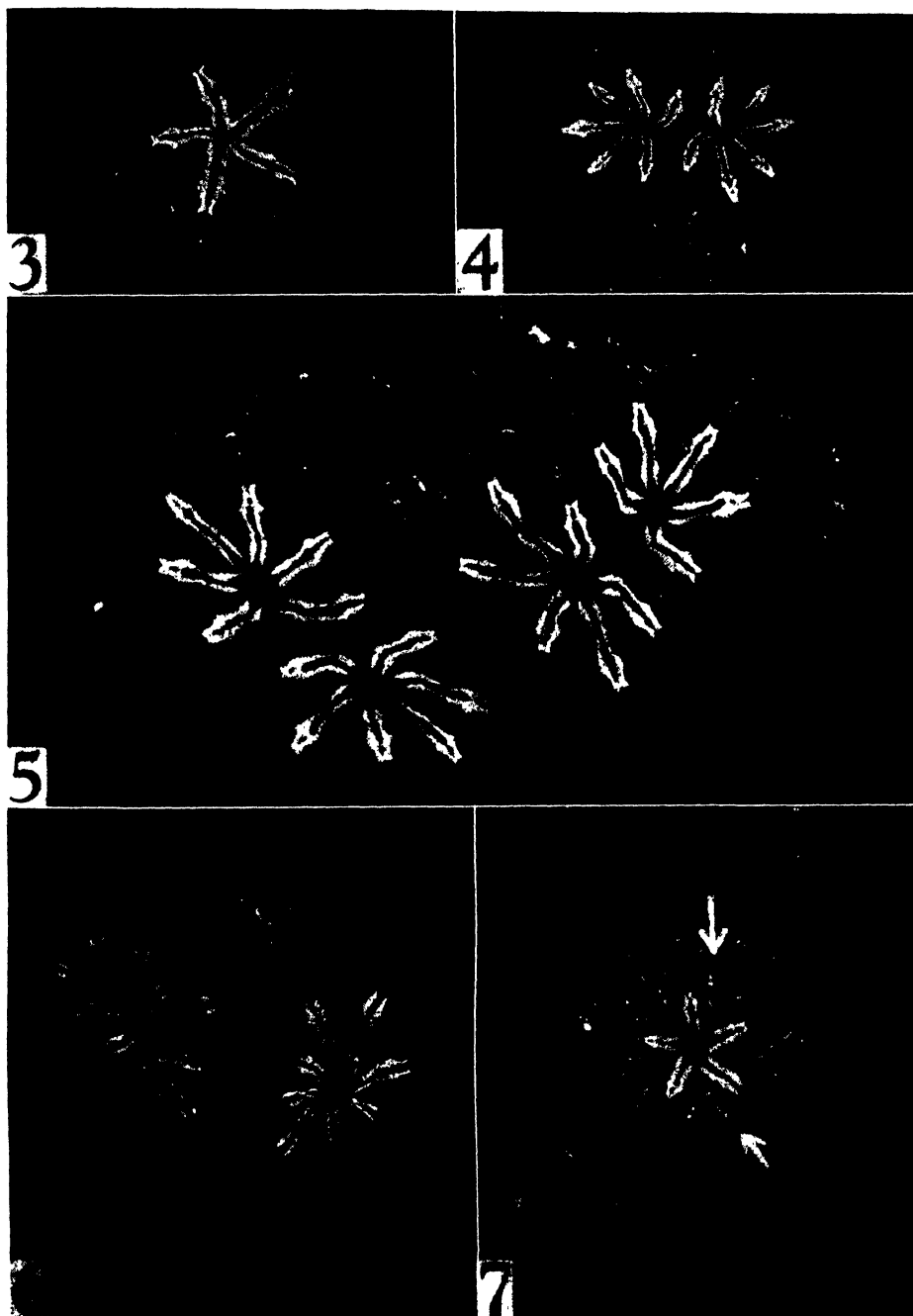


PLATE I

The asexual bud which will develop into the first generation blastozoid is already present in the tadpole before its emergence from the parent zooid (Pizon, 1893). It is readily visible on all oozoids the second day after the tadpole has attached (1Z in Figs. 1f, 1g, 1h and 2a). Sometime during the third day the bud begins to grow rapidly; a few scattered reflecting cells may then be present in the bud.

**Fourth day (Fig. 2b).** Both siphons are now visible in the first generation blastozoid. Reflecting cells are quite definitely restricted to the region immediately surrounding the siphons, with a few cells between the siphons and even to either side of the mid-dorsal line in some colonies. Meanwhile in the oozoid there is a tendency for the reflecting cells to become rearranged in part of the intersiphonal band, leaving spaces free from reflecting cells; such a space is forming in colony No. 38, although it is very small. In other oozoids the rearrangement of reflecting cells on the dorsal surface is much more extensive, especially near the oral siphon, serving to split that part of the originally single intersiphonal band into a double band. Occasionally reflecting cells may disappear first from other parts of the band. In three of the colonies the intersiphonal band appears somewhat contracted; in one colony some of the reflecting cells have been swept into the ampullae<sup>4</sup> by way of the circulatory system. The buds of the second generation blastozoids (2Z) are already present. Typically one bud forms on the right side and one on the left of the first blastozoid (although sometimes only one bud forms, in which case it is always on the right side; in other cases there may be three buds, in which case two are always on the right and one on the left).

**Fifth day (Fig. 2c).** The oozoid has almost completely degenerated. Usually the remnants of the oozoid are so heavily pigmented that it is impossible to identify whatever traces of the old intersiphonal band may still exist. Numerous reflecting cells arising from the disintegration of the intersiphonal band of the oozoid are now circulating freely in the blood stream and these can be traced as they move through the blood channels surrounding the siphons and in the area be-

<sup>4</sup> Ampullae are terminal enlargements of the colonial blood vessels. Eight of them are present in the oozoid (Grave and Woodbridge, 1924); these progressively increase in number during the formation of a colony. In an adult colony they are found nearly everywhere, but are situated principally at the periphery. Several functions are attributed to them by Bancroft (1899); they act as storage reservoirs for the blood, they aid in blood propulsion since they are contractile, they act as organs for the secretion of test matrix and they probably function as respiratory structures.

## PLATE II

All figures are photographs of the dorsal surface of the colonies taken by reflected light. Magnification ca. 7X.

**FIGURE 8. Colony 11. Age 33 days.** 6Z zooids are in maximum and permanent contraction and their intersiphonal bands are disintegrating. Reflecting pigment cells are accumulating in the ampullae at the periphery. 7Z zooids are appearing between the ampullae and the degenerating zooids and the oral extremities of their intersiphonal bands are forming (arrows). This is the second stage in the destruction of the intersiphonal patterns. On the 34th day the 7Z zooids are well formed with distinct intersiphonal bands.

**FIGURE 9. Colony 35. Age 33 days.** 7Z zooids are functioning and their intersiphonal bands are becoming double. Note the remains of the 6Z zooids at the center of the systems. Note also that reflecting pigment cells have almost completely disappeared from the peripheral ampullae.

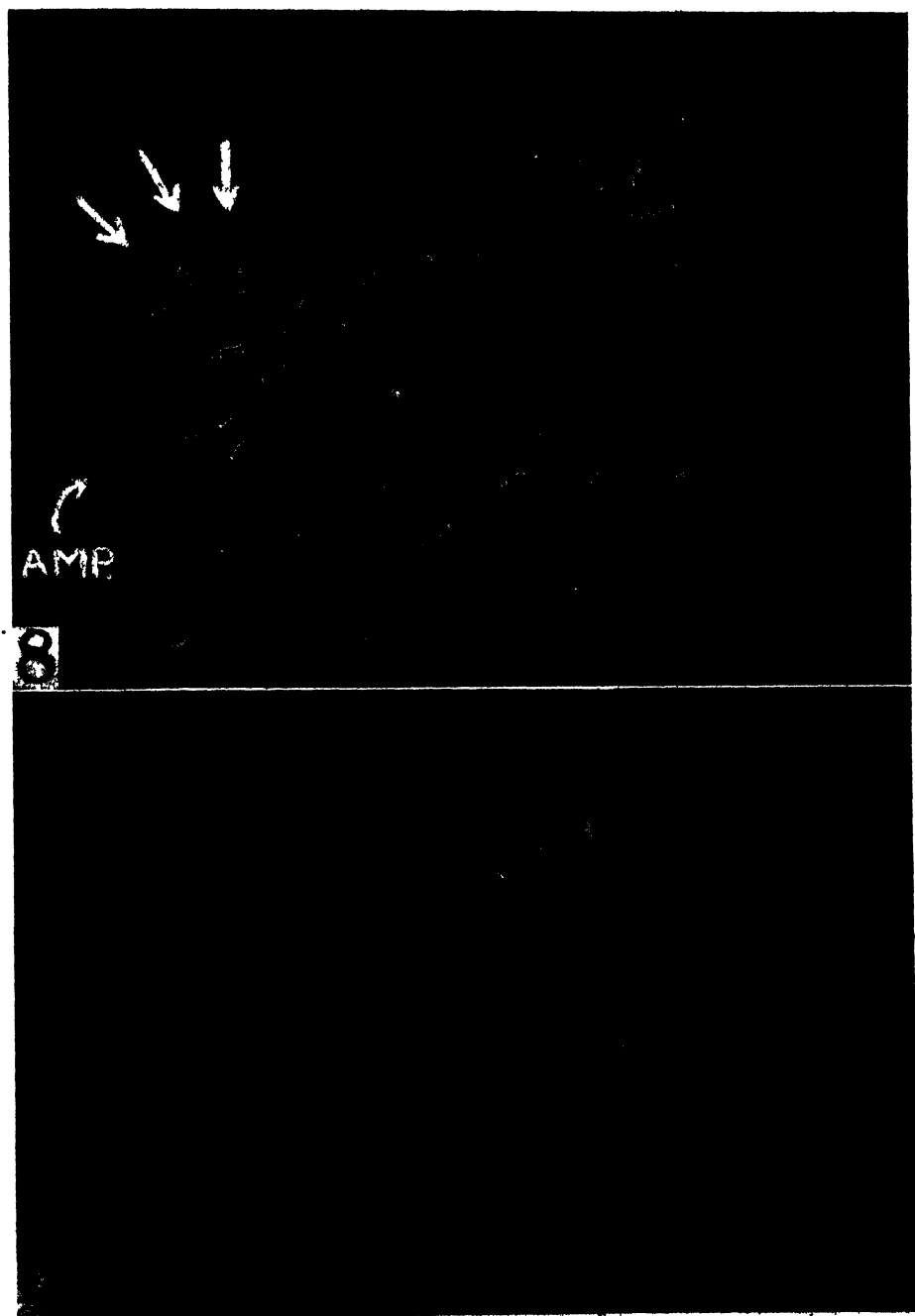


PLATE II

tween the siphons of the first blastozoid. Simultaneously the intersiphonal band of pigment cells in the first blastozoid rapidly becomes more distinct by the addition of more reflecting cells in this region. Typically a solid band of reflecting cells is established between the two siphons; this band may be almost rectangular in shape, or it may be slightly irregular. Within the band there may be one or even two spaces free from reflecting cells. Some of the reflecting cells from the degenerating intersiphonal band of the oozoid can also be followed as they are carried through the blood channels of the second generation buds. While these buds remain small the circulating pigment cells may become trapped in them temporarily which accounts for the presence of a few scattered reflecting cells early in the establishment of each generation of buds; these tend to disappear later when blood channels enlarge allowing them to move on.

In order to compare the changes in the other colonies with those described for colony No. 38 reference should be made constantly to Table I which summarizes the relationship between changes in the intersiphonal bands of pigment cells and the major changes involved in asexual reproduction in all colonies studied. For example, on the fifth day the oozoid is undergoing degeneration in 23 colonies (i.e., in 59 per cent of all colonies). In ten of these reflecting cells are contained in the vascular channels and ampullae; circulating reflecting cells are also visible in two other colonies in which the oozoids are completely destroyed. Thus pigment cells are circulating in 12 colonies (30.7 per cent). In 13 colonies (33.3 per cent) intersiphonal bands are forming in first generation blastozoids and they are all single bands (in the other 26 colonies the reflecting cells in the first generation blastozoids are too scattered to constitute a definite intersiphonal band). Second generation buds are beginning to grow in 23 colonies (59 per cent). In general the observations given below refer only to colony No. 38; in order to ascertain how characteristic these changes are for all the colonies reference must be made to Table I.

**Sixth day** (Fig. 2d). All traces of the oozoid have disappeared but some reflecting cells are still present in the ampullae. The intersiphonal band in the 1Z zoid is well formed; within it there is one space free from reflecting cells. In several other colonies one or two spots free from reflecting cells are now visible within the intersiphonal bands, and in two colonies this tendency for reflecting cells to disappear from the midline of the intersiphonal band is so marked that the band is now classified as double (Table I). The 2Z buds are growing, and in some colonies the 2Z buds are sufficiently developed that they in turn bear buds which will become the third generation (3Z) zooids.

**Seventh day** (Fig. 2e). A second space free from reflecting cells has appeared in the midst of the intersiphonal band of the 1Z zoid. Oral siphons are beginning to appear in the 2Z zooids; some scattered reflecting cells are also present in these zooids. 3Z buds are present. Reference to Table I shows that very few oozoids are actually undergoing degeneration at this time and that reflecting cells are circulating in very few colonies. Perhaps the most striking change in the appearance of the intersiphonal bands is clearly demonstrated in Table I, viz., that in many colonies the intersiphonal bands which were originally single are now definitely becoming double due to the disappearance or rearrangement of reflecting cells at one, two, three or four spots along the length of the band.

**Eighth day** (Fig. 2f). The intersiphonal band tends to be almost double throughout its length. Typically the disappearance of reflecting cells spreads inwards from each end of the band, so that the last part of the band to become double is the part midway between the two siphons. Oral siphons are distinctly present in 2Z buds, and in some colonies the atrial siphons have also appeared (see Table I). Reflecting cells are present in many second generation buds, but they are seldom restricted sharply to the siphonal areas.

*Destruction of Intersiphonal Bands in First Generation Blastozooids and Their Formation in Second Generation Zooids; Establishment of First Intersiphonal Patterns*

**Ninth day** (Fig. 2g). The first generation blastozooid is undergoing degeneration; reflecting cells are circulating vigorously. In all colonies where 1Z zooids are degenerating reflecting cells are circulating (Table I). The 2Z zooids have grown rapidly; they possess both oral and atrial siphons and their intersiphonal bands are well developed; three clear spots are present in the band of one zooid and two in the other. The buds which will develop into the third generation zooids are just beginning to enlarge. A few scattered reflecting cells are appearing in these 3Z buds.

**Tenth day** (Fig. 2h). All traces of the first generation blastozooid have disappeared; however, reflecting cells are still present in the ampullae. The degeneration of 1Z zooids is still underway in many colonies (Table I). Although the two atrial siphons of the two 2Z zooids are only closely approximated in colony No. 38 they have fused in nine colonies to form a common cloaca, thus establishing a system of zooids for the first time in the life history of these colonies. In one of these colonies consisting of three zooids two atrial siphons have fused, but the third one is still separate. In some colonies the buds which will develop into the fourth generation zooids have already made their appearance (Table I).

**Eleventh day** (Fig. 2i). Reflecting cells are no longer present in the circulatory system of most colonies. The intersiphonal bands of the 2Z zooids are almost completely double, especially at the atrial end of the bands. The atrial siphons of the 2Z zooids have now fused to establish a common cloaca. Therefore the individual intersiphonal bands are now united at their atrial ends for the first time to form a true intersiphonal pattern. The buds which will develop into the third generation blastozooids are enlarging; in many colonies these buds contain scattered reflecting cells with no special distribution. The buds which will become the fourth generation zooids are now present.

**Twelfth day** (Fig. 2j). The intersiphonal bands continue to approach the condition of completely double bands, especially at their atrial ends; consequently the intersiphonal pigment patterns tend to consist of double bands in each blastozooid (as in Pl. I, Figs. 3 and 5). In four colonies one or more of the 2Z zooids are beginning to degenerate prematurely; in two of these the 2Z zooids had first joined a common cloaca before degeneration began (in contrast to Pizon, 1893, 1899a, and 1900, who believes that one of the primary causes of premature degeneration of zooids is the failure to join a common cloaca with other zooids). Oral siphons are appearing in 3Z buds; 4Z buds are present, but are not growing.

*Destruction of Intersiphonal Bands in Second Generation Blastozooids and Their Formation in Third Generation Zooids*

**Thirteenth day** (Fig. 2k). 2Z zooids with almost completely double bands are still present, but reflecting cells are appearing in the circulatory system. The 2Z zooids are beginning to degenerate in a number of colonies; in some only one or two zooids are degenerating while others are still normal; in several the zooids are strongly contracted, but actual degeneration has not yet begun. 3Z zooids are considerably larger, oral siphons are present in them, and scattered reflecting cells are also visible, especially around the oral siphons. 4Z buds have not yet started to grow.

**Fourteenth day** (Fig. 2l). Second generation zooids are undergoing degeneration, as is the case in many other colonies (Table I). In two colonies only one of the second generation zooids has started to degenerate; in another colony one of the 3Z zooids is also degenerating. The 3Z zooids have their siphons well formed and the atrial siphons have already fused to form a common cloaca. The intersiphonal bands are well developed; some of them are nearly double from their earliest appearance. The 4Z buds are enlarging.

**Fifteenth day** (Fig. 2m). The 2Z zooids are completely absent although they are still undergoing degeneration in several other colonies. Almost no change has occurred in the appearance of the intersiphonal bands. The buds which will become the fourth generation zooids are growing rapidly and 5Z buds have now appeared.

**Sixteenth-seventeenth days** (Fig. 2n). The 3Z zooids form a well-defined system with almost completely double intersiphonal bands of pigment cells. One colony is striking in that the band is double in one of the two zooids, but practically non-existent in the other. Oral siphons are just appearing in 4Z buds. In two colonies some of the 3Z zooids are degenerating prematurely.

*Destruction of Intersiphonal Bands in Third Generation Blastozooids and Their Formation in Fourth Generation Zooids*

**Eighteenth day** (Fig. 2o). 3Z zooids are undergoing degeneration and reflecting cells are circulating. Intersiphonal bands are forming rapidly in 4Z zooids, and they are double from their earliest appearance. The atrial siphons of the 4Z zooids have not yet joined to form a common cloaca. 5Z buds are beginning to grow.

**Nineteenth day** (Fig. 2p). All traces of the 3Z zooids are now lacking. The atrial siphons of the 4Z zooids have not yet joined for cloaca formation. 5Z buds are growing rapidly and 6Z buds have appeared.

**Twentieth-twenty-first days** (Fig. 2q). Beyond the nineteenth day daily observations were made only on ten colonies (9c, 11, 13, 23, 30b, 35, 38, 41, 44a and 46b); therefore the observations summarized in Table I beginning with the twentieth day are based only on these ten colonies unless otherwise indicated. Colony No. 38 has well developed 4Z zooids; a distinct intersiphonal pattern is now present since the atrial siphons have joined to form a common cloaca with the almost completely double intersiphonal bands radiating outwards from it.

*Destruction of Intersiphonal Bands in Fourth Generation Blastozooids and Their Formation in Fifth Generation Zooids; First Appearance of More Than One Intersiphonal Pattern in a Colony*

**Twenty-second day** (Fig. 2r). 4Z zooids are undergoing degeneration; reflecting cells are circulating strongly. The 5Z zooids do not possess atrial siphons as yet; their intersiphonal bands are almost completely double from their first appearance. These fifth generation zooids are distributed into three groups indicative of the three systems of zooids which they will constitute later; two groups of four zooids each are separated somewhat from the remaining zooids which, though widely separated from each other, are destined to form only one system.

**Twenty-third–twenty-sixth days** (Fig. 2s). 5Z zooids are now well developed; all the intersiphonal bands are completely double and are radiating outwards from three common cloacas, i.e., the colony now consists of three systems of zooids and therefore of three intersiphonal patterns. 6Z buds are enlarging.

*Destruction of Intersiphonal Bands in Fifth Generation Blastozooids and Their Formation in Sixth Generation Zooids*

**Twenty-seventh day** (Fig. 2t). The 5Z zooids are undergoing degeneration and reflecting cells are circulating; all the thirty-two 6Z zooids have had double intersiphonal bands almost from their earliest appearance. These zooids are distributed in four groups indicative of the four systems they will establish, although no common cloacas have yet formed; zooids are distributed in groups of seven, eight, eight and nine.

**Twenty-eighth–thirty-first days** (Fig. 2u). Four well-defined systems of 6Z zooids are now present, each with a common cloaca and an intersiphonal pattern. The observations recorded in Table I for the twenty-ninth day are based on all 39 colonies, i.e., on the 10 colonies on which daily observations have been made, as well as on the 29 colonies which have not been examined since August 19. The observations recorded beyond the twenty-ninth day are again based only on ten colonies.

*Destruction of Intersiphonal Bands in Sixth Generation Blastozooids and Their Formation in Seventh Generation Zooids*

**Thirty-second day** (Fig. 2v). Colony No. 38 has changed from the sixth to the seventh generation of zooids; all of the new intersiphonal bands are almost completely double; the zooids are already arranging themselves into eight groups, but only a few atrial siphons have fused as yet. There is a total of 61 zooids.

**Thirty-third–thirty-fifth days** (Fig. 2w). The 7Z zooids are now arranged in eight distinct systems, each with its distinct intersiphonal pattern consisting of double intersiphonal bands.

## DISCUSSION

### *Literature*

Attention has frequently been called to the brilliant and varied coloring of the flower-like groups of ascidiozooids which constitute Botryllus colonies (M'Intosh, 1901; Herdman, 1925). According to Webb (1939), "It appears that all brightly

coloured species owe their colour to pigment cells which are morphologically blood cells, though in most cases they do not participate much in the general circulation but become more or less permanently lodged along the walls of the peripheral vessels" (p. 505). For the most part, however, only incidental observations have been recorded concerning intersiphonal bands of pigment cells. Pallas (1774) illustrates several *Botryllus* systems in his Plate IV, Figs. 2, 3, 4 and 5, and double intersiphonal bands are clearly indicated. Krohn (1869b) in his explanation of his Plate XIV, Fig. 2 calls attention to "Breiter kreideweisser Pigmentstreifen zwischen den beiden Leibesöffnungen." Van Name (1910) states that "During life, . . . , the zooids, especially their anterior ends and dorsal portions, and the bulbs of the test vessels, are marked with a light-colored pigment, which mostly disappears after death" (p. 352). In more detailed descriptions of *Botryllus* colonies the presence of some sort of intersiphonal bands has been noted in numerous instances. This is especially true of *Botryllus violaceus* (Milne-Edwards, 1842). In this species (?) Giard (1872) notes (p. 621), "Les deux orifices sont reliés entre eux par des lignes plus ou moins larges, d'une coloration qui varie entre le blanc pur et le jaune brunâtre en passant par toutes les teintes intermédiaires." Concerning this same species (?) Alder and Hancock (1912) state (p. 70), "the thorax from near that [oral] aperture downwards [is] marked with a double line of opaque white, broadest above and ending on the margin of the tubular common orifice; these lines [are] sometimes yellowish and occasionally broader and confined more exclusively to the base." In *Botryllus smaragdus* Giard (1872) mentions the presence of "Lignes radiales d'un jaune plus ou moins vif, parfois à peine indiquées" (p. 626) and Pizon (1899a) describes "des lignes radiales jaunes qui s'étendent, avec une largeur et des nuances très variables, sur la face supérieure de la branchie" (p. 396). Similar radial lines extending from the oral to the atrial siphons have been described by Giard (1872) in *Botryllus auro-lineatus* and *Botryllus morio*, by Alder and Hancock (1912) in *Botryllus miniatus* and by Van Name (1931) in *Botryllus primigenus*. Of these authors only Pizon definitely states that the pigment granules constituting the intersiphonal bands are contained within special cells called chromatocytes, and that these granules are about  $1\ \mu$  in size and are animated by Brownian movement. Van Name (1902) also notes that in the closely related *Botrylloides nigrum* similar white pigment is contained in opaque oval cells of the same size as those containing the ground color.

The only extensive observations concerning intersiphonal bands in *Botryllus* are recorded by Bancroft (1903). He refers to these bands in one of two ways: as dorsal double bands, or as two parallel white bands extending from cloaca to branchial orifice; he also speaks of the blastozooids possessing such bands as white-striped zooids. Although he does not state so specifically he realizes that the pigment involved is contained in specific pigment cells. He believes that the position of these bands does not depend on that of blood vessels. His paper deals to a considerable extent with color variations in *Botryllus*. His comments concerning variations in intersiphonal bands will be incorporated into the discussion below.

#### *Repeated Destruction and Formation of Intersiphonal Bands of Pigment Cells. During the Development of a Colony*

The intersiphonal band which develops in the oozoid is usually a solid band of cells between oral and atrial siphons. This band arises rather rapidly during

metamorphosis of the tadpole and undergoes few changes while the oozoid persists, except that it sometimes tends to become double at its oral extremity. This transformation of single intersiphonal bands into double bands occurs quite slowly in the oozoids and is rarely completed before the oozoid degenerates, so that single or at best partially double intersiphonal bands are characteristic of oozoids at their highest state of development. By the third day after attachment the first generation blastozoid is growing rapidly and scattered reflecting cells are present in it. By the fourth day both of its siphons are indicated and its reflecting cells are quite definitely restricted to the region immediately surrounding the siphons, with a few scattered cells between the siphons. The oozoid then begins to degenerate and the intersiphonal band of the oozoid is destroyed. The pigment cells set free from the intersiphonal band are then present in the circulatory channels and are carried passively by the blood stream. Such degenerative changes occur in the oozoids of the majority of colonies on the fifth day (Table I). Simultaneously intersiphonal bands are forming in the first generation blastozoids, and they are all single bands. 2Z zooids are just beginning to grow rapidly, but they possess neither oral nor atrial siphons as yet. 3Z buds are not present. On the sixth day fewer oozoids are degenerating; most of the colonies have single intersiphonal bands forming in 1Z zooids, but in a few colonies these bands are transforming into double bands. 2Z zooids are growing rapidly, although they still possess neither oral nor atrial siphons. 3Z buds are now appearing. By the seventh day degeneration of oozoids is nearly completed and reflecting cells are circulating in few colonies. The most striking change has been the transformation of almost all single intersiphonal bands into double bands. Oral siphons are just appearing in 2Z zooids and 3Z buds are now present in almost all colonies. By the eighth day the first generation blastozoids are in their optimal state of development. The characteristics of this highly developed condition are as follows: (a) almost no reflecting cells are circulating, indicating that oozoids have all degenerated some time ago and that first generation blastozoids have not yet started to degenerate; (b) the intersiphonal bands of first generation blastozoids are almost all double, a condition which is usually realized only when the zooids are fully developed; (c) all 2Z zooids are growing rapidly and both oral and atrial siphons are forming in them; (d) 3Z buds are present in all colonies.

Then, on the ninth day the 1Z zooids begin to degenerate, accompanied by the appearance of reflecting cells in the circulatory system once more, and accompanied also by the onset of rapid growth of 3Z zooids. Degeneration of first generation zooids and their intersiphonal bands is at a maximum on the tenth day. Intersiphonal bands are forming in 2Z zooids, and note especially that there are almost as many colonies with double bands as with single bands at this time. For the first time the atrial siphons of 2Z zooids are fusing in some colonies to establish a common cloaca and thus to establish a system of zooids within each colony, thereby allowing establishment of the first intersiphonal patterns. 4Z buds are beginning to appear. By the twelfth day the 2Z zooids have attained their maximum development. The characteristics of this highly developed condition of the colonies are the same as those when 1Z zooids were best developed, viz., few reflecting cells are circulating, almost all intersiphonal bands are double, all 3Z zooids are growing rapidly, and 4Z zooids are present in almost all colonies. In addition atrial siphons of 2Z zooids have fused to form common cloacas in most colonies.

Degeneration of 2Z zooids begins on the thirteenth day, reaching its maximum on the fourteenth day. Most of the intersiphonal bands forming in 3Z zooids are single at this time. The atrial siphons of 3Z zooids are beginning to fuse to establish common cloacas. 3Z zooids reach their maximum development on the seventeenth day. The new intersiphonal pattern characteristic of the colony at this time is more complex than previously since it consists of more intersiphonal bands. Again the characteristics of the colonies at this moment of maximum development are the same as at the time of maximum development of 1Z and 2Z zooids.

3Z zooids then begin to degenerate and 4Z zooids attain their maximum development on the twenty-first day. Then the 4Z zooids degenerate and 5Z zooids reach their maximum development on the twenty-sixth day. These in turn degenerate and 6Z zooids attain maximum development on the thirty-first day. The 6Z zooids then degenerate and 7Z zooids are in their most highly developed condition on the thirty-fifth day. 5Z, 6Z and 7Z zooids are characterized by double intersiphonal bands almost from their earliest appearance. Thus under laboratory conditions a period of 4 to 5 days elapses between the highest state of development of one generation of zooids (and its intersiphonal bands) and the highest state of development of the next generation (and its intersiphonal bands).<sup>5</sup>

During each brief period of four to five days the intersiphonal bands undergo an orderly series of stepwise transformations. a. The pigment cells which constitute a new intersiphonal band first appear near the oral siphons. They are fairly scattered at first, but very soon they form a solid band of cells in the dorsal midline extending from oral to atrial siphons. b. The originally solid bands of reflecting cells then begin to separate lengthwise so that each intersiphonal band shows indications of becoming double throughout its length (Pl. II, Fig. 9). These bands progressively become more completely double (Pl. I, Fig. 4) until they are distinctly double (Pl. I, Fig. 3). The pigment cells then reflect light to the maximal extent and the particular generation of zooids involved has then attained its highest state of development. Bancroft (1903) also recognized that these intersiphonal bands are progressively built up. Pizon (1899a) realized that the intersiphonal bands change with time, but he gave no description of the changes. c. The most radical change in the intersiphonal patterns then occurs. They assume a very irregular appearance (Pl. I, Fig. 6) as compared with their extremely regular appearance at the time of their greatest development (Pl. I, Figs. 3 and 5). This irregularity is due to the contraction of some of the intersiphonal bands, due in turn to the contraction of the zooids involved. At a given instant some intersiphonal bands are almost completely contracted, others only partially, but an instant later different bands become contracted and those formerly contracted are then somewhat relaxed, with the result that the intersiphonal patterns are rapidly changing their configuration. The intersiphonal bands then undergo maximum and permanent contraction (Pl. II, Fig. 8), and at this time the intersiphonal bands are destroyed, i.e., the reflecting pigment cells constituting them are released from the

<sup>5</sup> This period of four to five days between the maximum development of successive generations of zooids agrees with the intervals established by Berrill (1935a). However, it should be emphasized, as Bancroft (1903) and Grave (1933) have already done, that growth of *Botryllus* is less vigorous in aquaria than in its natural habitat. In colonies growing in the Eel Pond, Grave observed that the interval between successive generations is reduced to 2 or 3 days.

band and pass into the blood channels between and around the siphons and degeneration of the zooids is underway. These circulating pigment cells are swept quickly into the ampullae (amp., Pl. II, Fig. 8) at the periphery of the colony and into the blood channels of the next generation blastozooids and can be traced as they circulate around and between the siphons of these new zooids. These reflecting pigment cells sometimes begin to circulate even before the atrial siphons have appeared in the new generation zooids, and they are usually circulating strongly when the degenerating zooids are still of adult size, are three-fourths that size, are one-half as large as adults, and even when they are completely reduced.

While the old intersiphonal bands are undergoing destruction new intersiphonal bands are forming in the new generation zooids (Pl. II, Fig. 8, arrows). Because of the almost perfect coincidence in the time of destruction of the intersiphonal bands in one generation of zooids and their establishment in the next generation it seemed certain that some of the circulating reflecting cells must become secondarily attached in the intersiphonal region of the next generation of zooids to participate in the formation of its intersiphonal bands. This seemed all the more certain in view of the fact that during the final rapid development of these new bands of pigment cells the reflecting cells disappear almost completely from the circulatory system (compare peripheral regions of Pl. II, Figs. 8 and 9). However, in some exceptional cases (Pl. I, Fig. 7) it is evident that new intersiphonal bands (arrows) can originate even before contraction and subsequent destruction of the bands of the old generation zooids has begun. This suggests that the first reflecting cells to appear in the intersiphonal region of the new zooids arise *in situ*. Whether all the pigment cells of the new bands arise locally, or whether they are augmented with cells from the intersiphonal bands of the degenerating zooids can be determined only by experimentally preventing the reflecting cells from the degenerating pigment bands from entering the new generation zooids, as well as by a careful histological study. Bancroft (1903) has recorded an observation of interest in this connection. In his studies on the fusion of *Botryllus* colonies he has observed that a single system may contain zooids derived from two different colonies; the zooids from one colony may possess well developed double intersiphonal bands whereas those of the other may possess no visible bands (Pl. XVII, Fig. 23). In his discussion (pp. 174-175) he states that "In the fused colonies the zooids may differ in color; and not the slightest tendency toward an equalization of this difference could be detected." This evidence would suggest that intersiphonal bands arise *in situ* or not at all; it suggests that even if reflecting pigment cells are present in part of a colony they can never participate in the formation of a pigment band in zooids which do not inherently possess one. If the reflecting cells from the degenerating pigment bands are not involved in the establishment of new intersiphonal patterns, perhaps they too are used as food material by the newly forming zooids; it is known that all other cells arising from degeneration of the zooids are so utilized.

Eventually the number of zooids in a colony has increased to the point where they can no longer gather around a single common cloaca. More than a single common cloaca then forms, and some zooids assemble around each with the result that the colony then consists of more than one system of zooids. Since the intersiphonal bands of one system constitute one intersiphonal pattern, the colony then has more than one intersiphonal pattern. As more and more generations of zooids develop the number of systems, and therefore the number of intersiphonal patterns,

progressively increases. Krohn (1869a and b) clearly recognized that as each new generation of buds matures, the old systems are constantly replaced by new ones which usually correspond with the parent system neither in their form nor in the number of single individuals composing them.

Thus it is evident that any Botryllus colony is characterized by a specific intersiphonal pattern or group of patterns for only a very brief period. Any given intersiphonal pattern is then rapidly destroyed within a few hours and an entirely different pattern or group of patterns is built up, only to be destroyed in turn. Moreover, it is evident that this progressive remodeling of the intersiphonal patterns within a given colony is intimately associated with the process of asexual reproduction, so intimately in fact as to verify the statement by Berrill (1941c) to the effect that there is a "rigorous correlation of the features characterizing any given stage, so that if the developmental stage of one feature is known the stage as a whole can be accurately defined" (p. 100).

#### *Variations in Intersiphonal Patterns When Different Colonies Are Compared*

##### **Some Colonies Lack Distinct Intersiphonal Patterns**

Some colonies are collected in nature in which the intersiphonal patterns are not strongly expressed even when the zooids are in their maximum state of development. It is interesting to inquire whether this weakness in expression of the pattern in certain colonies is merely due to lack of proper environmental conditions, whether it is due to an unhealthy condition of the colony, whether it is a temporary condition characteristic only of that particular generation of zooids which is functioning at that instant, or whether it is a permanent characteristic of that particular colony appearing in all generations of zooids. If such a weakness of expression is a constant characteristic in certain colonies, perhaps it is genetically determined.

Two days after attachment of the larvae considerable variation is evident when the intersiphonal bands of different oozoids are compared. Some oozoids exhibit a *strong* intersiphonal band, i.e., there is a broad, almost solid band of reflecting cells between the two siphons; others possess a *medium* band, i.e., there is a rather narrow or irregular band consisting of relatively few reflecting cells; still others possess a *weak* intersiphonal band, i.e., there are only a few reflecting cells near the dorsal midline with no obvious arrangement into a band. In 15 of the colonies on the sixth day the reflecting cells are not concentrated into a distinct intersiphonal band in 1Z zooids. Of these, 11 were derived from oozoids originally classified as possessing *weak* bands and 4 from oozoids with *medium* bands; all of the oozoids whose intersiphonal bands were originally classified as *weak* (and not omitted from the observations recorded in this paper) are among these 15 colonies, but 6 colonies derived from oozoids whose bands were originally classified as *medium* are not in this group. No blastozooids derived from oozoids which exhibited *strong* intersiphonal bands are found in this group. On the eighth day 14 of the 15 colonies still do not have the reflecting cells arranged into a definite intersiphonal band, and no distinct intersiphonal band is formed before the 1Z zooids degenerate. By the eleventh day 25 colonies possess well developed intersiphonal bands in 2Z zooids, but such bands are lacking in 14 colonies. All of the colonies derived from oozoids originally possessing *weak* bands are among these 14, as

well as 3 from oozoids whose intersiphonal bands were originally classified as *medium*; not a single colony derived from an oozoid exhibiting *strong* intersiphonal bands is to be found in this group. Moreover no distinct bands appear in these 14 colonies before the 2Z zooids degenerate. On the sixteenth day, although distinct bands are present in 4Z zooids in 22 colonies, no such bands exist in 15 colonies, and all colonies originally exhibiting *weak* bands are included among these 15. On the twenty-ninth day three colonies originating from oozoids with *weak* intersiphonal bands possess no distinct intersiphonal bands in 5Z zooids; two colonies are undergoing the transformation from fifth to sixth generations and therefore the expression of the intersiphonal bands could not be determined; six possess no distinct bands in 6Z zooids. Two other 5Z colonies and three 6Z colonies are now classified as possessing no distinct intersiphonal patterns; these were derived from oozoids originally classified as possessing *medium* intersiphonal bands.

Thus all colonies in which only *weak* intersiphonal bands form in the oozoid stage form only *weak* bands throughout their development, at least through the seventh generation of blastozooids. This weakness of expression of the intersiphonal bands is therefore a constant characteristic throughout the developmental history of certain colonies. On the contrary, oozoids which possess well developed (*strong*) intersiphonal bands develop into colonies which always have well developed bands. This difference between *weak* and *strong* expression of intersiphonal bands is therefore a constant one and is probably genetically determined. In the present study no attempt was made to determine whether the tadpoles which give rise to oozoids with *weak* intersiphonal bands are derived only from adult colonies in which no distinct bands occur, or whether they can come from adults possessing distinct bands. However, Bancroft (1903) has recorded some observations of interest in this connection. He collected tadpoles from three mother colonies. The tadpoles from the first mother colony gave rise to a large number of colonies called Family I; tadpoles from the second mother colony established Family II; and Family III originated from tadpoles which emerged from the third mother colony. He observed that double bands formed only in Family II and Family III, and the mother colonies of both these families had well developed dorsal bands. He also noted that in Family II two colonies developed dorsal double bands, whereas two had no trace of them. This may indicate that colonies with *strong* intersiphonal bands can be established only from tadpoles which emerge from mother colonies with well developed bands; however, colonies with *weak* intersiphonal bands can also come from tadpoles emerging from colonies with well developed bands. Should there prove to be a genetic basis for this difference between *strong* and *weak* expression of intersiphonal bands, it appears likely that the *strong* expression will prove to be dominant.

The category of *medium* as applied to the classification of intersiphonal bands appears to be an artificial one, since colonies derived from oozoids classified as *medium* are subsequently classified in part with the group lacking distinct bands in later generations, but mostly with the group possessing such bands. Thus the group of oozoids classified as *medium* is probably a heterogeneous group, part of its members actually belonging among the *weak* group, but showing the maximum development of the bands to be found in this group, and the rest belonging to the *strong* group, but showing only a minimal development of the bands. The classification of *medium* is therefore discarded as of no significance.

### The Number of Zooids in a Given Generation Is Not Constant

#### *Due to differences in the number of buds per zooid*

Usually throughout the developmental history of any colony the number of zooids progressively increases and accordingly as each new generation of zooids becomes mature, the intersiphonal pattern of the colony becomes more complex than when the previous generation of zooids was present. However, the complexity of the pattern is not the same in all colonies even when they are in the same stage of development. This is evident if the intersiphonal patterns of three of the colonies illustrated in Plate I are compared. These 3 colonies are all in the same stage of development, i.e., sixth generation zooids are functioning in each, but in the colony illustrated in Figure 3 there are only 5 zooids in the 6Z generation, in the colony illustrated in Figure 4 there are twelve 6Z zooids, while in the colony illustrated by Figure 5 there are twenty-three 6Z zooids. Obviously even when colonies of the same stage of development are compared variations in their intersiphonal patterns still exist due to differences in the number of zooids, which is due mainly to differences in the rate of budding in different colonies. A question then arises concerning the factors which determine the number of buds produced by any zooid. Is the number of buds produced by a single zooid purely a fortuitous response to the environment, varying from generation to generation within any given colony? Or, is the number of buds produced by each zooid constant from generation to generation in a given colony and variable only when different colonies are compared? Any regularity in budding within one colony may possibly indicate that the budding pattern is a genetic characteristic of a colony.

According to Pizon (1893) both lateral walls of the atrium of the tadpole undergo a localized thickening sometime before the tadpole escapes from the parent. Such a thickening is the first step in the development of an asexual bud. Thus budding in the oozoid begins bilaterally, but the thickening on the left is always much less accentuated than the one on the right. By the time the tadpole has escaped from the parent the bud on the left has entirely disappeared, whereas the one on the right has developed sufficiently that the beginnings of a pharynx and two atrial sacs are recognizable. He notes that the intestine in the oozoid is crowded to the left and he believes that the pressure exerted by the intestine against the atrial wall most likely inhibits the morphogenesis of the left bud at an early stage. Most ascidiologists agree that the oozoid gives rise to a single bud on its right side, although Giard (1872) and Jourdain (1886) believe that the oozoid generally produces two first generation blastozooids.

According to Herdman (1925) bilateral budding is then the rule in the blastozooids, so that theoretically the colony should consist of a number of zooids represented by some power of two. Pizon (1899a and 1900) also regards as normal the production of two buds, one on the right and one on the left of the parent, although in many instances only one bud develops, and in these cases he states that the single bud may be either on the right or left side (1893). However, Berrill (1941b) has noted that there is usually a size difference between bud primordia on the right and left sides, the one on the right being the larger. Moreover, Oka (1892) states that whenever an equal number of buds is developing on the right and left sides of the parent those on the left are always somewhat retarded. It is therefore not surprising that Oka finds that whenever a single bud

is produced, it is always on the right side. Whenever one bud is lacking Pizon always finds a feeble diverticulum of the atrial wall where that bud should have developed, indicating that budding of the blastozoid is always bilateral initially regardless of whether both buds actually continue to develop. Krohn (1869a) and Della Valle (1882) on the contrary regard the production of a single bud as the rule among blastozoids and the production of more than one bud per zooid as the exception. However, Krohn recognizes that two buds can form, in which case one is always on the right and the other on the left.

Oka (1892) has also observed that many times the original atrial thickening is not entirely used up in the production of one bud leaving a portion of the budding zone unused. Sometimes this remnant of budding material gives rise to a second bud on that same side of the parent; such a second bud is always younger in its developmental stage than the first bud developed on that side of the body. Della Valle (1882) also recognized that when more than one bud develops on the same side of the body the first one to appear is always in advance of the second in its stage of development. Pizon (1893) has observed one young colony of *Botryllus* in which each blastozoid carried two buds on each side; he states that the same peribranchial thickening on each side produced the two adjacent diverticula. Oka (1892) records that in the *Botryllus* colonies found along Japanese coasts it is not unusual to find zooids producing as many as six asexual buds. If an even number of buds is produced, there will be the same number of buds on each side of the body; if an odd number is formed, there is always one more on the right than on the left. According to Berrill (1941c) double buds are more likely to develop in early generations of zooids before sexual reproduction begins as well as in later generations when large zooids capable of reproducing sexually are present, rather than in intermediate stages.

Thus it is obvious that there are bilateral bud primordia in both the oozoids and blastozoids, but that there is an asymmetry of budding capacity with the right side usually the more productive. Most of the above information concerning the budding pattern is based on isolated observations or on a study of a very small number of colonies. It seems worthwhile to follow the budding patterns of the colonies studied in this paper in an attempt to determine whether any generalities concerning the budding pattern can be formulated.

When all the colonies are examined on the sixth day of development 6 possess only one 2Z bud (2ZR), 21 possess two 2Z buds (2ZR and 2ZL), and 12 possess three 2Z buds, two on the right side (2ZR and 2ZR') and one on the left (2ZL). When the second generation zooids are examined on the tenth day to determine the number of 3Z buds per zooid, it is noted that in some colonies each zooid produces only one 3Z bud; in others some zooids produce one 3Z bud and others two such buds; in other colonies all zooids produce two buds each; in still others some zooids produce two 3Z buds and others three 3Z buds. In order to decide whether the number of 3Z buds per zooid in any given colony bears any relationship whatsoever to the number of 2Z buds per zooid earlier produced by the same colony, the data may be arranged advantageously in a checkerboard table (Table II). From this table it is evident that three of the six colonies (50 per cent) which produced only one 2Z bud per zooid also produce only one 3Z bud per zooid; moreover, an additional one of these six colonies (17 per cent) produces one 3Z bud per zooid in at least some of the zooids. Ten of the 21 colonies (48

TABLE II

Comparison of the number of buds per zooid in different generations of the same colonies

|                                       |        | No. of 3Z buds per zooid on tenth day |                 |             |                  | No. of 4Z buds per zooid on seventeenth day |                 |            |                  |           | Av. no. of 6Z zooids per colony on twenty-ninth day |
|---------------------------------------|--------|---------------------------------------|-----------------|-------------|------------------|---|-----------------|------------|------------------|-----------|---|
|                                       |        | 1 bud                                 | 1 bud or 2 buds | 2 buds      | 2 buds or 3 buds | 1 bud                                       | 1 bud or 2 buds | 2 buds     | 2 buds or 3 buds | 3 buds    |   |
| No. of 2Z buds per zooid on sixth day | 1 bud  | 3<br>(50%)                            | 1<br>(17%)      | 2<br>(33%)  |                  | 1<br>(17%)                                  | 2<br>(33%)      | 3<br>(50%) |                  |           | 6.17  |
|                                       | 2 buds | 5<br>(24%)                            | 3<br>(14%)      | 10<br>(48%) | 3<br>(14%)       | 2<br>(9.5%)                                 | 10<br>(48%)     | 8<br>(38%) |                  | 1<br>(5%) | 12.23   |
|                                       | 3 buds |                                       | 7<br>(58%)      | 2<br>(17%)  | 3<br>(25%)       | 3<br>(25%)                                  | 7<br>(58%)      | 1<br>(8%)  | 1<br>(8%)        |           | 20.90   |

per cent) which produced two 2Z buds per zooid also produce two 3Z buds per zooid; moreover, six additional colonies (28 per cent) produce two 3Z buds per zooid in at least some of their zooids. Thus, in these two groups of colonies there appears to be at least some slight tendency for colonies to produce the same number of second and third generation buds per zooid. However, those 12 colonies which originally produced three 2Z buds per zooid appear incapable of maintaining this rapid rate of budding, and tend to produce only two 3Z buds per zooid instead (in 17 per cent of these colonies there are two 3Z buds on each zooid, but in 58 per cent there are two 3Z buds on only some zooids and one 3Z bud on others, and in 25 per cent there are two 3Z buds on some zooids and three 3Z buds on others). Pizon (1899a) has noted a similar tendency towards reduction in the number of buds per zooid following a generation in which three buds per zooid was the rule, and he suggests that this reduction serves to compensate for the excessive budding of the earlier generation. In his case the reduction was to even less than two buds per zooid.

Similarly, in order to see whether the number of 4Z buds per zooid bears any relationship whatsoever to the number of 2Z buds per zooid produced by the same colonies these data may be added to the checkerboard table. In some colonies on the seventeenth day there is only a single 4Z bud per zooid; in others some zooids produce one bud, others two; in some colonies each zooid produces two 4Z buds; in others some of the zooids produce two 4Z buds, others three 4Z buds; in one colony each zooid produces three 4Z buds. Of the six colonies originally producing one 2Z bud per zooid only one colony (17 per cent) produces a single 4Z bud per zooid. Fifty per cent produce two buds per zooid, and an additional two colonies (33 per cent) produce two buds per zooid in at least some of their zooids. Thus, by the fourth generation produced by asexual budding these colonies appear to be approaching the ability to produce two buds per zooid. However, if each 4Z zooid then produced two 5Z buds, and each of these in turn produced two 6Z buds, these six colonies should average eight zooids in the sixth generation. Instead they actually average only 6.17 zooids, and therefore slightly less than two buds per zooid must be the rule in giving rise to fifth and sixth generation zooids.

Of the 21 colonies originally producing two 2Z buds per zooid eight (38 per cent) produce two 4Z buds per zooid, but ten (48 per cent) produce two 4Z buds on only some of their zooids. Thus the ability to produce two buds on each zooid is no longer maintained rigidly in these colonies. If each zooid did continue to produce two buds up to the 6Z generation, we would expect to find thirty-two 6Z zooids. Instead we find on the average only 12.23 6Z zooids, indicating a budding rate of about 1.5 buds per zooid beyond the formation of 3Z buds. Those twelve colonies originally capable of producing three 2Z buds per zooid have almost lost this ability by the fourth generation of blastozooids. Only one colony (8 per cent) produces three 4Z buds in some of its zooids; seven colonies (58 per cent) produce two 4Z buds in some of their zooids and in three colonies (25 per cent) only one 4Z bud per zooid is produced. Thus, in these colonies, less than two buds per zooid now seems to be the rule in producing fourth generation blastozooids. If two buds per zooid continued to form to give rise to 4Z, 5Z and 6Z generations, we would expect to find forty-eight 6Z zooids per colony; instead we find only 20.9 6Z zooids per colony on the average, or roughly a budding rate of 1.5 buds per zooid after the 3Z zooids are established.

Grave (1933) has emphasized that the growth rate of different *Botryllus* colonies varies greatly even in their normal environment, but he made no attempt to analyze these differences in terms of the number of buds produced per generation of zooids. He has observed one colony which developed from a single larva on June 20 to 3000 individuals on July 20; he states that the number of individuals in this colony doubled every two or three days. In this colony the number of buds per zooid must have been at least two to account for such rapid growth; however, he states explicitly that he selected as examples only the most proliferative colonies; therefore in most colonies the budding rate must be less than two buds per zooids.

Therefore, although colonies differ in the number of 2Z buds produced per zooid, this difference appears to be only temporary and is not maintained in later generations, at least not under laboratory conditions. Instead, in later generations all colonies tend to produce on the average 1.5 buds per zooid. Nevertheless, the initial difference in the number of 2Z buds per zooid is sufficiently great that even though the budding rate then tends to become equalized in all colonies, the number of zooids present in the 6Z generation will be small in colonies which originally produced only one 2Z bud per zooid (6.17), will be about twice as great in colonies originally producing two 2Z buds per zooid (12.23), and will be about three times as large in colonies originally producing three 2Z buds per zooid (20.9). Consequently the number of 2Z buds per zooid is an important factor in accounting for differences in the intersiphonal patterns in later generations even when colonies in the same stage of development are compared. The factors which account for the differences in vigor, which in turn allow one, two, or three 2Z buds to form on each zooid, are not known. If they are due to genetical differences the action of heredity in controlling the number of buds per zooid appears to be limited to controlling bud formation only in the establishment of this one generation. These observations seem to be based on a study of more colonies than those examined by Berrill (1935c); his observation that the first bud (1Z) gives rise to a second bud (2Z) and that to a third bud (3Z) and the third to a bud on each side may hold for

a particular colony, but it is certainly not the characteristic budding pattern of the majority of colonies.

*Due to premature atrophy of developing buds*

In addition to differences in the actual number of buds the possibility that some of these buds may degenerate prematurely before reaching the adult stage must also be considered as a limiting factor in controlling the number of zooids in a colony and therefore the complexity of the intersiphonal patterns of the colony. Pizon (1899a) has observed that frequently a considerable number of buds are arrested in their development and begin to regress. Some disappear very early, at the moment when they are still small simple vesicles which are beginning to produce herniations on the flanks of their parents. Others regress much later when they have already attained one-fifth to one-fourth adult size. Regression even occurs prematurely in some large zooids which have the internal organs almost completely formed.

Pizon (1893, 1899a and 1900) regards the failure of the bud primordia to produce buds as well as the premature atrophy of such buds once they have started to develop as due primarily to nutritive deficiencies. Abortions occur when buds are too closely pressed against the older zooids or against the substrate of the colony, thereby constricting the blood channels and preventing the entrance of the blood stream from parent into bud. Moreover he notes that such atrophy is more frequent in colonies in early stages of formation and suggests that this may be due to the fact that at first all of the energies of the newly forming colony are directed toward elaboration of the test with little left over to enable buds to develop profusely. He particularly emphasizes that isolated zooids or very small systems are more likely to atrophy than large systems, and he suggests that it is necessary that a zooid participate in the organization of a system if it is to survive. Oka (1892) also suggests that crowding or lack of it probably determines whether or not a bud will develop to maturity, but he emphasizes that the left bud primordium is more sensitive to adverse conditions than the right, and is less likely to survive. However, there are other examples of premature degeneration where no apparent causes are to be discerned. During the first seven generations of blastogenesis studied in this present paper there have been few examples of premature atrophy of zooids once they have started to develop. Where such atrophy occurred there was no indication of crowding. Moreover, as noted earlier (p. 83), some zooids degenerate prematurely even after they have become adults and even though they are associated with other zooids within a fairly large system, so participation in system formation is not in itself a guarantee that premature degeneration will not occur.

*Due to persistence of parent zooids after their buds attain the adult state*

Another factor which can affect the number of zooids present in a colony at a given stage of development is whether the parent zooids always degenerate as soon as their buds become capable of functioning, or whether they sometimes persist to function simultaneously with their buds. The results recorded in this paper are in complete agreement with those of Pizon (1893) who states that when one generation attains the adult state, the preceding generation degenerates, and that two generations of zooids never arrive at the adult state simultaneously. However, several authors (Delle Valle, 1882; Bancroft, 1903; and Berrill, 1935b) have re-

ported that two generations of zooids can function side by side. If this actually does happen, the number of zooids and accordingly the complexity of the intersiphonal patterns would be correspondingly increased.

#### *Due to fusion of adjacent colonies*

Yet another factor which could influence the number of zooids in a colony and accordingly the intersiphonal patterns is the possibility that adjacent oozoids or older colonies may fuse indistinguishably. No such fusion was witnessed in the present work because every effort was made to prevent it by destroying adjacent colonies whenever there was a possibility of their fusing. Herdman (1925) suggests that large colonies need not necessarily arise from a single oozoid since several larvae may attach close together and form colonies which fuse so indistinguishably that even within one system some zooids may be derivatives of one oozoid, whereas the others come from another oozoid. Pizon (1900) has recorded an unquestionable case of such fusion. Bancroft (1903) has carried out an extensive study of the process of fusion and he has demonstrated that although unrelated colonies generally do not fuse, fusion can occur between some of the larvae derived from the same mother colony providing they become attached close together. Thus, any colony whose developmental history is unknown may conceivably be the derivative of more than one oozoid.

### **The Formation and Geometrical Form of Systems and of Intersiphonal Patterns**

As indicated in the introduction each system consists of from 2 to 23 blastozooids, with an average of 8.3 blastozooids per system in 200 systems examined in colonies collected at Woods Hole. Obviously the intersiphonal pattern will vary according to the number of zooids involved in its formation. There is no opportunity for a system to form until the second generation zooids are well developed, since only a single zooid is present in the oozoid and first generation blastozooid stages. However, if there are two or more second generation or later generation zooids their atrial extremities approach one another and fusion of the separate atrial openings occurs so that the colony then possesses a central common cloaca about which the zooids are radially arranged with their separate oral siphons at the periphery. The originally separate intersiphonal bands then radiate from the common cloaca and the net result is the first establishment of an intersiphonal pattern. Sometimes, especially in older colonies, the zooids have to move through considerable distance to participate in the establishment of a system. Pizon (1899a) has observed zooids in less than one day make half to almost a complete turn in order to bring their atrial extremities into contact with those of other zooids. Sometimes the formation of a common cloaca occurs in stepwise fashion, i.e., two or more zooids may form one common cloaca, and several may form another, but later these two cloacas fuse to become a single opening. Bancroft (1903) states that there is evidently some attractive force, the nature of which is entirely unknown, which causes zooids to join into a system.

There appears to be a maximum number of zooids which can constitute a single system. According to Pizon (1899b) and Herdman (1925) when blastozooids become too crowded against each other and no space remains for additional zooids

around a single cloaca one or more blastozooids become crowded outwards away from the cloaca and come to lie at the periphery, each such isolated zooid constituting a starting point for the formation of an additional system. Oka (1892) also emphasizes that when additional systems are first established they contain only a small number of individuals, usually three or four and many times only two.

Several features of system formation should be analyzed because of the changes in the intersiphonal patterns which are involved. Actually the existence of an intersiphonal band on each blastozooid greatly simplifies the task of observing the relationship of zooids to one another during the formation of systems. a. Is the form of a system (and accordingly of the intersiphonal pattern) identical whenever the same number of zooids forms one system? Is it identical even when different generations of zooids are compared? What factors affect the form of the intersiphonal pattern? b. What is the relationship between the number of zooids in a colony and the number of systems formed? Is there any inherent tendency for a colony to have a certain number of zooids per system, or is the number of zooids comprising a system purely a matter of chance? c. When an additional system is established, does it actually consist at first of a very small number of zooids?

When only two zooids constitute a system they typically occupy less than  $90^\circ$  of the space available around the cloaca and the form of the intersiphonal pattern is invariably the same, viz., the form of a letter V in which the angle between the intersiphonal bands is less than  $90^\circ$ . This is true whether the zooids involved are second, third or fourth generation zooids. Beyond the fourth generation there are usually more than two zooids per system, although in some colonies only a single zooid is present. If three zooids constitute a system the form of the intersiphonal pattern is slightly different depending upon the generation of zooids involved. If the zooids are of the second generation they occupy only  $90^\circ$  of the space available around the cloaca and an angle of approximately  $45^\circ$  is formed between adjacent intersiphonal bands. If the three zooids are third or fourth generation zooids they occupy  $180^\circ$  of the available space around the cloaca and an angle of  $90^\circ$  is formed between intersiphonal bands. If the three zooids involved are fifth or sixth generation zooids they tend to occupy all of the available space around the cloaca and the angle between adjacent intersiphonal bands usually increases to  $120^\circ$ . When systems consist of four zooids they tend to occupy all available space around the cloaca and adjacent intersiphonal bands usually form an angle of  $90^\circ$  with one another, although if third generation zooids are involved, less than  $360^\circ$  of the space is filled and the angle is correspondingly less than  $90^\circ$ . If there are five to ten zooids per system they are arranged radially around the cloaca filling all available space, with equal angles between adjacent intersiphonal bands regardless of the generation under consideration (Pl. I, Figs. 3 and 7). Whenever there are eleven zooids or more in a system the cloaca becomes oval shaped and the system itself is oval in outline instead of circular and the intersiphonal pattern becomes less of a perfect radial pattern (Pl. II, Fig. 9, the system on the right).

Several factors are important in controlling the form of the system. First, whenever possible a zooid tends to be in contact with another zooid on each side and throughout as much of its length as possible. Usually such

an arrangement is not possible until more than three zooids are present in a system, because three zooids or less are not usually enough to occupy all of the space around a common cloaca; thus the number of zooids available for system formation is a second factor determining the form of the system. The size of the zooids in the different generations is a third factor involved in the form of a system. Bancroft (1903) and Berrill (1941b and c) have both noted that the size of zooids increases progressively, with each generation. The larger the zooids involved, the fewer needed to occupy all the space available around the cloaca. If the above three factors affecting the form of systems are kept in mind it is possible to predict the form of any system in any colony providing the number of zooids and the generation of zooids is known. These factors exercise full control over the form of the system only when the system is isolated, i.e., only when there is a single system in a colony. When more than one system is present in the same colony the form of the systems is always modified due to crowding of zooids at the contact points between adjacent systems (Pl. I, Figs. 4 and 5).

Certain generalizations can be made concerning the relationship between the number of zooids in a colony and the number of systems formed. The maximum number of zooids which can constitute a single system is most commonly 14 or 15. Occasionally by the time 11 or 12 zooids are present the colony breaks up into two systems. If the colony consists of 16 to 22 zooids two systems are usually formed. If there are 23 to 30 zooids there are usually three systems. When the colony consists of 31 or 32 zooids four systems are usually present. When 52 to 55 zooids are present simultaneously they are most commonly distributed among six systems, and eight systems are usually found when as many as 61 or 62 zooids are present. Whenever the colony consists of more than one system of zooids the actual number of zooids within any one system seems to be determined primarily by chance. On the average there are eight zooids to a system, but there may be from four to fifteen in the colonies whose development has been followed. As far as can be determined there are no inherent differences between colonies in the number of zooids which constitute a system. This means that the number of intersiphonal bands of pigment cells which enter into the formation of any single intersiphonal pattern is purely a matter of chance. It would seem that if each new generation of zooids is larger than the previous one the number of zooids constituting a system should decrease with increased age of the colony. Such a tendency to decrease the number of zooids per system is not evident during the first seven generations of asexual reproduction. However, Bancroft (1903) has noted that in older colonies the number of zooids per system is reduced to three or four on the average.

The observations of Oka (1892), Pizon (1899b) and Herdman (1925) concerning the method of formation of additional systems seem open to question. According to Herdman (pp. 204-205), "When there is no longer room for all the ascidiozooids around the original cloaca, one or more may be pushed out into the surrounding test. Here they continue to form lateral buds and so found new systems." This would suggest that if more zooids are present than the maximum number which can be contained within one system, any extra zooids are crowded out singly from the old system and each of these

isolated zooids, by budding, then establishes a new system in the following generation. This implies that in order to form an additional system at least two generations of zooids must be involved: in the first generation one or more zooids become crowded out of the old system; in the second generation the buds developed from the zooids isolated in the previous generation actually establish the new system. It implies, moreover, that at the time an additional system is established the colony always consists of one large system plus the one or more very small newly established systems. Actually any such single zooids if crowded out of a system probably degenerate prematurely (see p. 96). Oka also emphasizes (p. 543) that "Ein neu gebildetes System enthält nur eine geringe Anzahl von Individuen, gewöhnlich deren drei oder vier, manchmal sogar nur zwei. Da aber bei jedem Auftreten einer neuen Generationen die Zahl der Individuen sich verdoppelt, so kommt es im Verlauf einiger Generationen zu einem typischen System von acht oder neun Individuen. . . ." However, elsewhere in his description Oka states (p. 542), "Nach dem Tod der Muttergeneration, welcher bald erfolgt, sieht man die jüngeren in ein System, häufiger aber noch in zwei gleich grosse oder ungleich grosse Systeme sich zusammenlagern." This latter statement of Oka alone accurately describes the formation of additional systems. Even before the death of the parent zooids the buds are roughly grouped according to the systems they will later form. And following the death of the parent zooids the newly formed zooids almost immediately become distributed into as many systems as are destined to form. There is no intermediate step during which all zooids first attempt to join around a single cloaca, followed by isolation of any extra zooids to establish smaller systems. Moreover, an additional system when it is first formed consists of a considerable number of zooids, Oka and Herdman to the contrary. In the ten colonies studied most extensively in this paper an additional system never contained fewer than four zooids when it was first established, and much more commonly it contained seven to nine zooids. Thus, when a colony contains sufficient zooids to form an additional system, the new system does not contain only the excess zooids which can no longer be accommodated in the old system, but rather the old system is replaced by two systems, each with approximately equal numbers of zooids.

### **Intersiphonal Bands as Taxonomic Characters**

Any attempt to use slight differences in the appearance of intersiphonal bands as a basis for classifying *Botryllus* into species should be regarded with caution. Unless the taxonomist is fully aware of the progressive transformations which the intersiphonal bands undergo and of the time relationship between these transformations and the stages of asexual reproduction errors could be introduced by comparing the appearance of intersiphonal bands in colonies in entirely different stages of development. Moreover, even when colonies of the same stage of development are compared there will be differences in the number of intersiphonal bands in an intersiphonal pattern, as well as differences in the number of intersiphonal patterns in the colony, but such differences are due seemingly to factors which are not of taxonomic

importance. And even the absence of intersiphonal bands in some colonies may indicate nothing more than a single gene difference between these and colonies which exhibit intersiphonal bands.

#### SUMMARY

1. The most striking feature of many *Botryllus* colonies is the localization of special light-reflecting pigment cells between the oral and atrial siphons of each zooid. Collectively these pigment cells constitute an intersiphonal band.

2. Individual zooids of a *Botryllus* colony are distributed radially around a common cloaca as a system of zooids. Consequently all the intersiphonal bands within one system constitute a star-shaped pattern called an intersiphonal pattern.

3. Any *Botryllus* colony is characterized by a specific intersiphonal pattern for only a very brief period of its existence. Any given intersiphonal pattern is then totally destroyed within a few hours and is replaced by an entirely different pattern or group of patterns. The formation and subsequent destruction of intersiphonal bands is described in detail for the oozoid and first seven generations of blastozooids in living colonies established from isolated larvae.

4. The progressive remodeling of the intersiphonal patterns is intimately associated with the changes involved in asexual reproduction. An interval of four to five days occurs between the maximum development of one intersiphonal pattern and the maximum development of the next. During this brief period a typical series of changes occurs in the appearance of a colony. a. The zooids become highly sensitive and contractile and the intersiphonal pattern becomes very irregular. b. Zooids then undergo maximum and permanent contraction, the intersiphonal bands disintegrate and the pigment cells are released into the circulatory system and are carried passively by the blood stream. c. Simultaneously the new generation of zooids is growing and the new intersiphonal bands form in them. At first these are solid bands, but they tend to split lengthwise until they become double intersiphonal bands, especially in older generations of zooids. d. Reflecting pigment cells then disappear from the circulatory system.

5. There is no evidence that the pigment cells released by the break-down of the intersiphonal bands of one generation of zooids can be utilized in the formation of new bands in the next generation of zooids.

6. If the oozoid develops a *strong* intersiphonal band of pigment cells (i.e., a broad, almost solid band of reflecting cells between the two siphons) only *strong* intersiphonal bands appear on all later generations of zooids; if it develops a *weak* intersiphonal band (i.e., only a few reflecting cells near the dorsal midline with no obvious arrangement into a band) only *weak* bands appear on all later generations of zooids. This difference between *weak* and *strong* expression is constant and is probably genetically determined.

7. Intersiphonal patterns vary in different colonies even though they are in the same stage of development. These variations are discussed in terms of a. differences in the number of buds per zooid in different colonies, b. the premature atrophy of developing buds in some colonies, c. the possible functional persistence of parent zooids after their buds become functional, d. the fusion of adjacent colonies.

8. The form of an intersiphonal pattern is determined by several factors: a. Whenever possible zooids tend to come into contact with another zooid on each

side and throughout as much of their length as possible; b. the number of zooids available for system formation is a determining factor; c. the size of the individual zooids likewise affects the form of the pattern. If these three factors are kept in mind it is possible to predict the form of any intersiphonal pattern providing the number of zooids and the generation of zooids is known. When more than one system is present in the same colony the form of the intersiphonal pattern is always modified due to crowding of zooids at the contact points between adjacent systems.

9. When a colony contains sufficient zooids to form more than one system the new system does not contain only the few excess zooids which can no longer be accommodated by the old, but rather the old system is replaced by two systems, each with approximately equal numbers of zooids. As far as can be determined there are no inherent differences between colonies in the number of zooids which constitute a system.

10. The appearance of the intersiphonal bands or patterns should never be used as a characteristic for classifying *Botryllus* into species unless one is fully aware of the range of variation that occurs even in the same colony and of the factors responsible for such variations.

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<sup>1</sup> For explanation of symbols see Supplement, vol. 84, no. 1, February 1943.

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## THE SIZE OF THE MESH OPENINGS IN MUCOUS FEEDING NETS OF MARINE ANIMALS

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### INTRODUCTION

In a former article (MacGinitie, 1937) the statement was made that "the slime net (*Urechis*) will entrap the smallest particles which are visible with the aid of an oil immersion lens." Experiments have just been completed which have determined much more accurately the size of the openings through which sea water is passed to strain from it the food ingested by animals using such nets. The results of these experiments have more than justified the statement quoted.

### THE FEEDING NETS

The mucous net of the echiuroid worm *Urechis caupo* (Fisher and MacGinitie, 1928; MacGinitie, 1935) is admirably suited to tests of the kind herein reported. In the mud flats of the west coast *Urechis* builds a U-shaped tunnel with two entrances eighteen to thirty-six inches apart, depending on the size and age of the animal, and with the lowest part of the tunnel from ten to eighteen inches beneath the surface. The openings of the burrows are somewhat constricted, and the animals, which live singly in a burrow, keep a current of water flowing through the burrow along the body by means of a more or less rhythmic peristalsis.

The slime tube for collecting food is secreted by a ring of mucous glands situated about one-sixth of the length of the body posterior to the proboscis. When the worm secretes a mucous tube, this ring of glands is pressed tightly against the periphery of the burrow to cause the mucus at the beginning of secretion to adhere to the sides of the burrow. As the animal backs up in its burrow more mucus is spun until the tube becomes four to six inches long. Then the spinning ceases, but the tube is allowed to remain attached to the secreting ring. This leaves the animal with its anterior end within a sack of mucus which is firmly attached to the "neck" as though by a drawstring; and the distal end of the mucous sack is firmly attached to the sides of the burrow. Thus all water flowing through the burrow must pass in at the open distal end and pass through the walls of the mucous sack before it can flow along the outside of the animal on its way through the burrow. As the water passes through this mucous net all microscopic and, as will be seen,

some ultramicroscopic, material is held by the net. After pumping water through the net for varying lengths of time, the end at the "neck" is slipped over the head and the entire tube with its load of food is swallowed.

The mucous food straining bag of the annelid *Chaetopterus variopedatus* (MacGinitie, 1939a) was also used for the purpose of testing size openings, though *Chaetopterus* is much more sensitive to strange substances than is *Urechis*. By using a liberal amount of time and patience, either animal becomes accustomed to the presence of proteins as they are introduced into the water currents passing through the burrows.

#### MATERIALS AND METHOD

Three substances of known molecular weight, and stained with T1824 Evan's Blue, were introduced into the mucous nets of both *Chaetopterus variopedatus* and *Urechis caupo* while the animals were feeding. The three substances and their molecular weights were: ovalbumin, 44,000; human serum globulin, 176,000; and hemocyanin (Palinurus), 450,000. The molecular weights given were taken from Cohn and Edsall (1943).

In the cases where excess dye was used for staining, the excess was taken up by dialyzing bags (Rawson, 1942-43), the proteins having been dissolved in sea water, 34 parts per thousand of salts. Rawson found that up to eight molecules of dye were bound by each molecule of globulin and that a 1:5 solution remained stable for one month. These figures were used also for ovalbumin and hemocyanin, though no experimental evidence was found for the use of eight molecules as a saturation limit, but absorption by cellophane dialyzing bags showed these limits to be fairly accurate.

#### RESULTS

The ovalbumin passed through the mucous net entirely. Enough serum globulin was held by the net to color it a decided blue, while no hemocyanin whatever passed through the mucus.

Molecular weights and shapes of proteins are subject to considerable uncertainty. Recently Cohn (1944) gives a molecular weight, length and equatorial diameter for human serum globulin of 160,000, 320 Å, and 36 Å, respectively. The  $a/b$  ratio is thus about 1/9. Human serum globulin has a frictional flow coefficient of 1.49 and hemocyanin of 1.23. Comparison of the two molecules on this basis and disregarding hydration, gives hemocyanin an equatorial diameter of approximately 90 Å, an  $a/b$  ratio of 1/5.

Ninety Ångströms seems to be a rather high figure, judging by what one can find in the literature relative to the diameters of protein molecules, but it is safe to say that the meshwork opening in a mucous feeding net is of a magnitude between 36 Å and 90 Å. However, since some human serum globulin was caught by the net while no ovalbumin was, and because all hemocyanin was entrapped, a logical conclusion is that the meshwork openings are quite close to 40 Å.

Solutions varying from two molecules of dye to one of protein to those with an excess of dye were used with hemocyanin. Since the dye T1824 has a molecular weight of 960 (Rawson, 1942-43), the molecular weight of any of the substances

used was not increased by more than 7,680. Human serum globulin was used in the proportion of five molecules of dye to one of the serum; thus the molecular weight was increased by 4,800. Allowing for this increase, the figure 40 Å was used instead of 36 Å, though the latter may be the more accurate figure.

The results described in this paper were the same for the mucous nets of both *Chaetopterus* and *Urechis*, and it is my opinion that all feeding nets where used by tunicates (MacGinitie, 1939b), pelecypods (MacGinitie, 1941), gastropods (in mss.), etc., are of like physical and chemical structure, though only actual tests with each animal will prove this to be true.

### DISCUSSION

There are several factors which preclude exact measurements, for example, uncertainty of molecular size, the shape of molecules, hydration, the shape of openings in the mucous network, surface conditions of both protein and mucoid molecules, rigidity of the mucus surrounding the openings, force or head of water flow, possible variation in the elasticity of the mucus, etc.

Molecular size should not show an error of more than plus or minus ten per cent or less, and the shape of molecules about the same or possibly more. It is difficult to conceive of straight openings from side to side of a mucous net .5 to 1 mm. in thickness. Where the mucous net remains attached to the body of *Urechis* it is about 3 mm. in thickness, and it was noted that the hemocyanin did not penetrate greatly beyond the inner surface. It did penetrate sufficiently, however, to become incorporated in the mucous network. It cannot be washed out, and later it is shown that no reaction takes place between the mucous tube and the hemocyanin. This indicates a sponge-like structure, and not openings running directly from side to side. Mehl (verbal communication) is of the opinion that the mucous molecules are long and slender. The last three factors, rigidity, force of flow and variation in elasticity, are more interdependent. The greater the rigidity, the more uniformly constant the openings, and the less affected by force of current.

Mucous nets are held under a certain amount of tension while the animal is feeding. When the net of *Urechis* is freed from the animal it at once collapses to one-half its original size and gradually draws up to much less than this if left free and immersed in sea water.

When *Urechis* is pumping water through a burrow of glass tubing for respiratory purposes (not feeding), and one end of the tube is held out of water the peristaltic movements can create a difference in head of four or more inches, which is equal to .144 lb. per square inch. Such pressures are never exerted upon a feeding tube. Under natural conditions perhaps but one-tenth of this amount of pressure is exerted upon the feeding tube.

A check was made to determine if on standing there was any reaction between the protein of the mucous tube and that of the substances used. Clean mucous nets were put in stained solutions of hemocyanin and human serum globulin and left for one-half hour periods, then centrifuged; no hemocyanin or serum globulin united with the tubes.

No dye has yet been found that will stain the pure mucus of a feeding net.

In one rechecking of these experiments, some hemocyanin (*Palinurus*) was used which had been precipitated by low pH and stored in a frozen state for about

six months. This hemocyanin had denatured to the extent that it all passed through the mucous net. It is known that hemocyanin denatures to a greater extent than most proteins, though all hemocyanins are not alike in their amount of dissociation. Neurath, Greenstein, Putnam and Erickson (1944), quoting Burk (1940), give a dissociation for *Limulus* hemocyanin of from 2,040,000 to 142,000 for 6.66 M urea treatment and to 69,000 for HCl followed by 6.66 M urea. Measurements were made on the basis of osmotic pressures. This is a dissociation to about 1/14 for the urea treatment and 1/30 for the combined HCl-urea treatment. The difference between 142,000 and 69,000 is due to the removal of copper by the acid.

The fact that all of the hemocyanin used in this rechecking experiment passed through is proof that its molecular size had been reduced, at least in one dimension, by a low pH or by freezing, to a size lower than that of the human serum globulin.

Another factor, of which there are perhaps many more than were listed above, that could influence the passage of long molecules through a network such as that just described is the possibility that by physical means the molecules may be

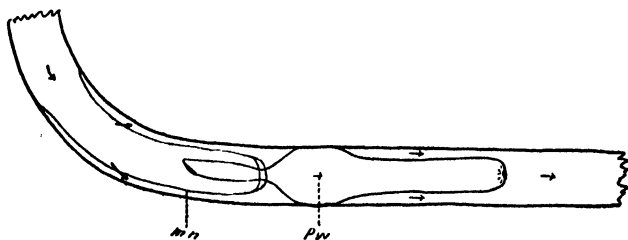


Diagram of *Urechis caupo* in a burrow, feeding. *m.n.*, mucous net; *p.w.*, peristaltic wave which drives water ahead of it. Other arrows indicate flow of water through the burrow.

oriented in such a way that they may enter the network openings of the mucus small end first. This could be due to Brownian movement or electrical charge or flow influence. Perhaps these feeding nets may be of value in helping us to solve such problems.

The mucous net has been used to estimate the extent of polymerization in certain proteins. It already has been shown that it is a test for denaturation of hemocyanin (*Palinurus*). It will also be useful in checking partial dissociation or denaturation or for possible adulteration by smaller protein molecules.

There still remains the possibility that other animals may secrete mucous nets with meshwork different in porosity from that of *Urechis* and *Chaetopterus*, and further experimentation may show that the mucous net may be stretched or allowed to contract to further increase its range of usefulness.

Considering the above findings, one is impressed with the efficiency of mucus as a net for straining food from water. The molecular size of decomposition products would preclude their use as food material, but all other organic materials, including protein molecules of a size greater than 40 Å in cross section could be used.

It is interesting to think about a substance, which when it comes in contact with water, takes up 200 or more parts by weight of the latter, yet remains a meshwork which allows water to pass freely through it. It is also interesting that in its wide

use among the marine invertebrates as a food gathering mechanism it is seldom if ever wasted, since it is necessarily swallowed with the food that it has entrapped.

I wish to express my appreciation to Dr. Linus Pauling, Dr. Dan H. Campbell and Dr. Albert Tyler for advice and materials, and to Dr. T. H. Morgan for reading the manuscript.

#### SUMMARY

1. A brief description is given of the food collecting net of the echiuroid worm *Urechis caupo*. Water flows freely through this mucous net.

2. By introducing into this mucous tube proteins of known molecular size, namely, ovalbumin, 44,000; human serum globulin, 176,000; and hemocyanin (Palinurus), 450,000, the size of the openings was found to be approximately 40 Ångströms.

3. Experimental uses for the mucous net are given, and other possible uses suggested.

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# STUDIES ON THE OXYGEN CONSUMPTION AND WATER METABOLISM OF TURTLE EMBRYOS<sup>1</sup>

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As a result of the early work of Bohr and Hasselbalch and the later experiments of Murray and others there is available a considerable body of information concerning the respiratory exchange of the chick throughout its embryonic life and, although vertebrate groups other than birds have been less extensively investigated, the respiratory characteristics of the embryos of various fishes and amphibians are also fairly well known. Only two investigations of the respiration of reptilian embryos have been reported however, and these deal with but a few stages.

Bohr (1904) carried on some experiments with the eggs of the snake, *Coluber natrix*, and found a decrease in respiration intensity with increasing age. The respiratory quotient as determined by Bohr was about 0.9 at all of the stages studied.

Zarrow and Pomerat (1937) studied four eggs of the Green Snake (*Liopeltis vernalis*) which were in late stages of development and also investigated the respiration of the young after hatching. They report that the absolute gas exchange of the eggs was of the same order of magnitude as that of the young snakes but the respiratory quotient of the hatched animals was significantly greater than that of the embryos.

The present experiments were undertaken with the purpose of providing closely spaced series of determinations of oxygen consumption of the reptilian embryo from the time the egg is laid until after hatching. Some data on changes in weight of the egg during development and on dry substance and fat content at different stages are also presented.

## MATERIAL AND METHODS

The eggs used in these experiments were obtained from four species of turtles; the Common Mud Turtle (*Kinosternon subrurum subrurum*), the Snapping Turtle (*Chelydra serpentina serpentina*), the Three-toed Box Turtle (*Terrapene carolina triunguis*), and the Eastern Painted Turtle (*Chrysemys picta picta*). One of these, the Three-toed Box Turtle, is a terrestrial form while the others are primarily aquatic. The Mud Turtles and Painted Turtles were collected from ponds in nearby Maryland, the Snapping Turtles were purchased from a dealer in Wisconsin and the Three-toed Box Turtles were sent to us from Monticello County, Arkansas, through the courtesy of Dr. Delzie Demaree.

Eggs were obtained by killing the adult females and removing the eggs from the oviducts. They were kept on moist cotton in open dishes in an incubator the

<sup>1</sup> We are indebted to the Permanent Science Fund of the American Academy of Arts and Sciences for a grant for the purchase of the Warburg vessels used in this investigation. The water bath used was made available through a previous grant from the Elizabeth Thompson Science Fund.

temperature of which varied between  $25.0^{\circ}$  and  $25.5^{\circ}$  C. Most of the eggs maintained under these conditions seem to have developed normally although a number of them died during the course of the long period which elapsed before hatching. Dead eggs had a tendency to become moldy on the surface and were, of course, discarded. Moreover it proved possible after some experience to ascertain, by candling the eggs, whether or not living embryos were present and in all experiments except those with very early stages this test was employed. A further check was provided by opening some of the eggs of the experimental series from time to time or by carrying them through to hatching. It is thus possible to state positively that all of the eggs which provided the data on oxygen consumption were living and apparently developing normally.

To obtain some information concerning changes in weight during development, some eggs of each species were weighed at intervals on an analytical balance. In all cases excess moisture was wiped from the surface before weighing and weights were taken to the nearest milligram.

Determinations of the oxygen consumption of eggs and hatched turtles were carried out with Warburg manometers equipped with flasks having about 120 cc. capacity. The  $\text{CO}_2$  was absorbed in the customary manner with KOH, the flasks were not shaken and the eggs were kept dry. In all experiments seven flasks were used simultaneously, one serving as a thermobarometer while the other six contained eggs. In the great majority of the experiments only a single egg was introduced into a flask. With very young stages at the beginning of our study, several eggs were used in each flask. Since, however, not all eggs develop normally it was later necessary to reject some of these experiments because, upon opening the eggs at a later date, one or more of a lot used in a single manometer proved to be infertile or abnormal. Since the rate of oxygen consumption of young stages is low, while that of older ones is high, the individual determinations were carried through periods of various lengths. With very young stages the usual period was 24 hours with two or three readings only, with older stages the period was 5 to 6 hours with hourly readings and with late stages 2 to 3 hours with half-hourly readings. In the case of very late stages of the Snapping Turtle the experimental periods were sometimes limited to  $1\frac{1}{2}$  hours because the oxygen consumption was so high as to preclude further readings with the manometers used. At the outset of the experiments it became clear that the temperature equilibration between the surroundings and the egg is very slow. When eggs were introduced directly from the incubator at  $25.0\text{--}25.5^{\circ}$  C. into the manometer flasks in a water bath at  $25.4 \pm 0.01^{\circ}$  C. the equilibration took at least two hours. It was therefore necessary to keep the eggs that were to be used for an experiment in the water bath over night. This was done by placing them on moist cotton in an open jar immersed in the water bath. Under such conditions an equilibration of 45 minutes before the manometers were closed proved to be sufficient.

Since it was necessary, in making the calculations of oxygen consumption, to know the volumes of the eggs used, a method was devised for measuring the volumes to the nearest 0.1 cc. The apparatus employed for this<sup>2</sup> was a wide-mouthed, glass-stoppered bottle with a 10 cc. graduated pipette fused in the stopper and a 20 cc. graduated syringe fused in the side of the bottle near its base. The bottle

<sup>2</sup> We are indebted to Mr. W. F. Simpson of this department for devising this apparatus.

was filled with water, closed and the water forced into the pipette by pushing the syringe to the zero mark. A reading was then taken on the pipette. The syringe was opened again to lower the water level, the stopper of the bottle was removed and the egg to be measured was inserted. The top was then replaced, the syringe was pushed to the zero mark and a new reading was taken on the pipette. The difference between this reading and the preceding gives the volume of the egg.

Determinations of the dry substance and fat content were carried out as follows. Two batches of young Snapper eggs (ten eggs each) and two batches of newly hatched Snappers (ten and seven turtles respectively) were dried to constant weight in an oven at 100–105° C. In the case of the eggs, the egg contents and the shells were dried separately. The fat content was determined according to the method of Kumagava and Suto (1908) with the slight modification used by Reinhard and von Brand (1944).

Large numbers of eggs of the Painted Turtle and Snapping Turtle were available and eggs of these forms were opened every few days to give closely staged series of preserved embryos. This makes it possible to correlate the oxygen consumption with both the age and the stage of development in these species. From the Mud Turtle and the Three-toed Box Turtle relatively few eggs were obtained and our series of normal stages for these animals are therefore incomplete.

## RESULTS

### *Developmental rate and incubation period*

In view of the paucity of published information concerning the embryology of the turtles used in the present work it is necessary, before discussing our experimental results, to give some general account of the developmental history of these forms.

The normal egg complement of a single female differs markedly in the four species. For the Mud Turtle we had only two adult females and these yielded four and three eggs respectively. Six adult Three-toed Box Turtles were used; four of these had only two eggs each in the oviducts and the other two had three eggs each. In a group of ten Painted Turtles the number of oviductal eggs ranged from two to eight, but most individuals had either five or six eggs and the average number was 5.4. The Painted Turtles used were all about the same size, having carapace lengths of 13.0 to 15.5 cm. Nine Snapping Turtles with carapaces 27.0 to 33.0 cm. long were opened. The smallest number of eggs found was 29, the greatest 61. The average yield for the nine animals was 44.5. This is of some interest in view of the fact that Pope (1939) records for the Snapper that "A female lays from twenty to about forty eggs at one time. Higher numbers (even up to seventy) are often given, but they probably are either sheer estimates or counts made in compound nests." It is, of course, not certain that all of the eggs found in the oviducts would be laid at one time or in one nest but our records do show that a single female may produce well over 40 eggs during one season.

In all of these species, and probably in turtles generally (Risley 1932, 1944), the eggs pass through the cleavage stages while still within the oviduct and are in early stages of gastrulation when laid. If the eggs are retained in the oviduct for unusually long periods they practically cease development during this time, possibly because of anaerobic conditions, and therefore eggs removed directly from the ovi-

duct are all at the same stage even though the dates of removal may be several weeks apart. Thus in our experiments, from a single lot of Snapping Turtles some females were killed on June 30 while others were retained until July 27, but the eggs of both were at the same stage of development when removed and were carried through to hatching with equal success.

At the temperature used in the present work ( $25.0\text{--}25.5^{\circ}\text{C.}$ ) the average times required for incubation were as follows: Mud Turtle, 76 days; Painted Turtle, 63 days; Snapping Turtle, 72 days. None of the Three-toed Box Turtle eggs were carried through to hatching but eggs opened after 70 days incubation contained well-formed turtles attached to yolk masses about 6.0 mm. in diameter. They would probably have required at least 10 more days before hatching. It should be pointed out that even under uniform environmental conditions there is some variation in the rate of development and the period of incubation. For example,

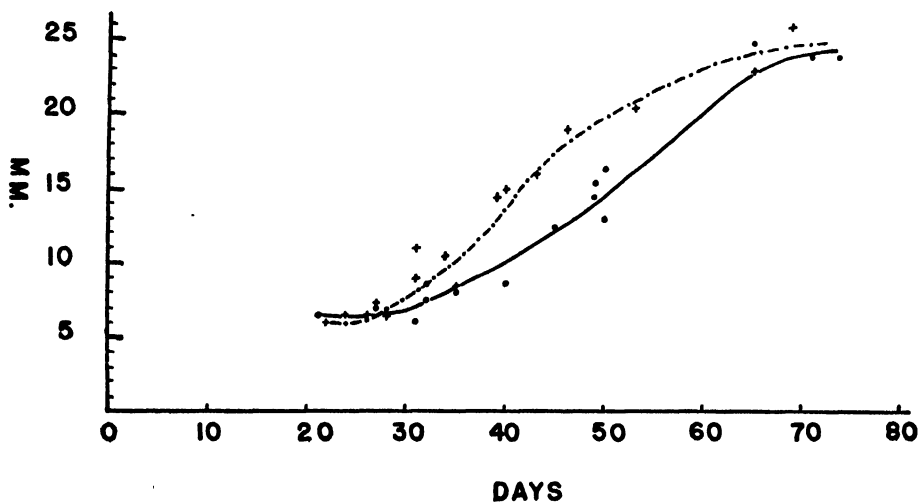


FIGURE 1. The increase in length of the carapace of the turtle embryo during the period of development. ● = Painted Turtle; + = Snapping Turtle.

in a single clutch of 8 Painted Turtle eggs the incubation period varied from 58 to 68 days and in a clutch of 61 Snapping Turtle eggs the limits of variation were 69 to 78 days.

As an indication of the relative rate of growth of the embryo at successive stages, measurements have been made of the series of preserved embryos of the Painted Turtle and the Snapper. Because of the difficulty of making reliable measurements of total length in these animals it has seemed best, for the present purpose, to use the length of the carapace. This can be measured accurately and does seem to furnish a reliable criterion of the rate of growth of the body as a whole. Such a procedure necessitates neglecting about the first twenty days of development, before the carapace is formed, but as will be seen it is the latter part of development which is of most interest in our results on the oxygen consumption.

Figure 1 gives curves for the increase in length of the carapace in the Painted Turtle and Snapping Turtle. Each point represents a single measurement. In

the case of the Painted Turtle all embryos in the series were from eggs kept in the incubator. Because of lack of space, the Snapper eggs used for the preserved series were kept in a laboratory the temperature of which varied but averaged about 25° C. The fact that these eggs hatched at the same time as did those in the experimental series of Snapper eggs which were kept in the incubator seems to justify the conclusion that the growth rate in the two lots was quite similar.

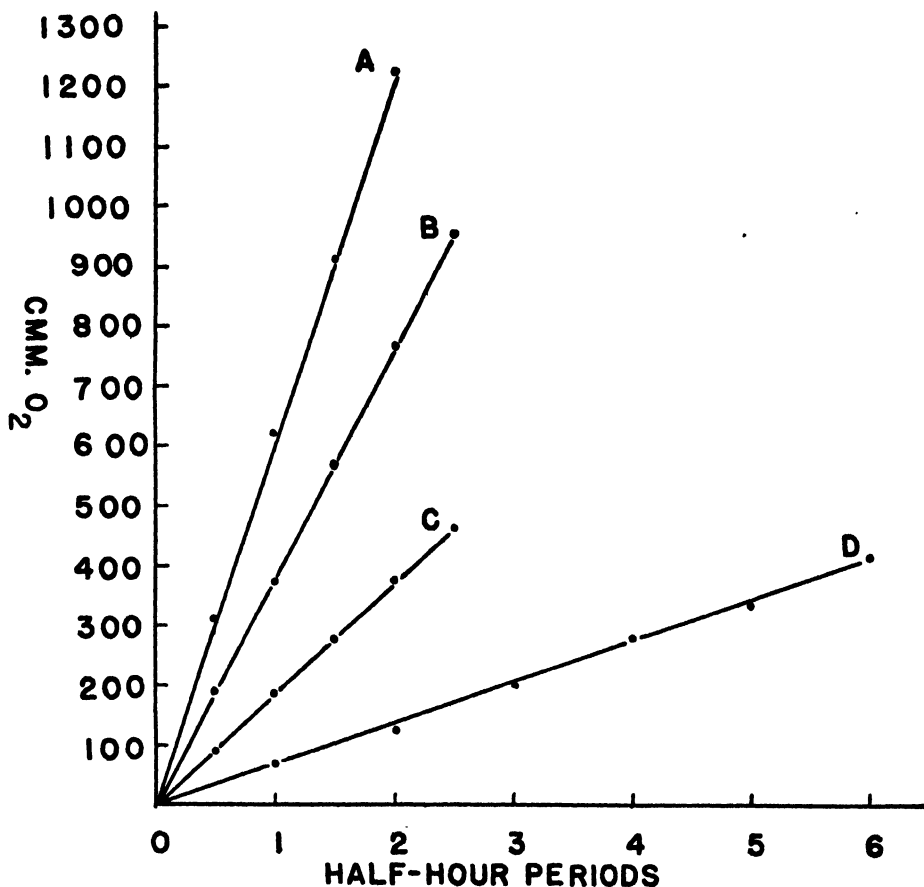


FIGURE 2. Oxygen consumption of four different specimens during consecutive periods of single experiments. A = newly hatched Snapping Turtle; B = Snapping Turtle egg, 42 days incubation; C = Mud Turtle egg, 45 days incubation; D = Painted Turtle egg, 23 days incubation.

It will be noted that the curves for the two species agree closely and indicate that the growth rate is relatively low during the early stages of development but rises rapidly after about the thirtieth day and then tends to remain nearly constant after the sixtieth day. The growth rate of the Snapping Turtle is somewhat higher than that of the Painted Turtle during the period from the thirtieth to fiftieth day however, so that its curve lies at a higher level than does that of the Painted Turtle

during this time. The general similarity of these curves to those for oxygen consumption will be noted later.

It should be pointed out that, although the length of the carapace at hatching was found to be the same in the Painted Turtle and Snapping Turtle, the Snapper hatchling is really much the larger animal for it is broader and bulkier than the young Painted Turtle. Thus the average weight of ten new-hatched Painted Turtles was 4.25 grams while the average weight of ten Snapper hatchlings was 7.18 grams. Clearly, therefore, the measurements of carapace length serve only

TABLE I

Oxygen consumption of developing turtle eggs, turtles in the process of hatching (hg) and newly hatched turtles (h). The figures are mean values representing cubic millimeters of oxygen per egg or organism per hour.

| Age in days | Three-toed Box Turtle | Mud Turtle | Painted Turtle | Snapping Turtle  |
|-------------|-----------------------|------------|----------------|------------------|
| 1           |                       |            | 18             |                  |
| 2           | 14                    |            | 32             |                  |
| 9           | 29                    | 13         |                |                  |
| 11          |                       |            |                | 35               |
| 12          |                       | 12         |                |                  |
| 15          |                       |            | 39             |                  |
| 16          |                       | 21         |                | 40               |
| 19          | 51                    |            |                |                  |
| 23          |                       | 31         |                | 55               |
| 24          | 80                    |            |                |                  |
| 32          |                       |            | 95             |                  |
| 34          |                       | 82         |                | 109              |
| 35          | 136                   |            |                |                  |
| 37          |                       |            | 133            |                  |
| 41          |                       |            | 185            |                  |
| 42          |                       |            |                | 332              |
| 44          | 180                   |            |                |                  |
| 45          |                       | 171        |                |                  |
| 49          |                       |            | 293            |                  |
| 50          | 287                   |            | 375            |                  |
| 52          |                       |            |                | 600              |
| 54          | 349                   | 386        | 526            |                  |
| 56          |                       |            | 456            |                  |
| 57          |                       |            | 396            |                  |
| 61          |                       |            | 463            |                  |
| 62          |                       | 387        | 552            | 798              |
| 63          | 428                   |            | 632 (hg)       |                  |
| 64          |                       |            | 427            |                  |
| 65          |                       |            | 476            |                  |
| 69          |                       |            | 555            | 751              |
| 70          | 350                   | 447        |                | 1,036 (hg)       |
| 72          |                       |            |                | 868 (hg) 641 (h) |
| 73          |                       |            | 581            |                  |
| 75          |                       | 418        |                | 617 (h)          |
| 76          |                       | 517 (h)    | 607 (h)        | 795 (h)          |
| 81          |                       |            | 748 (h)        |                  |
| 83          |                       | 437 (h)    |                |                  |
| 90          |                       | 541 (h)    |                |                  |

to give an indication of relative growth rates at different periods and do not tell us the absolute rate of growth. Since, however, our primary interest in relation to the oxygen consumption experiments is in the relative rate of growth, the data derived from the length measurements seem sufficient for our purpose.

### *Oxygen consumption*

When the precautions outlined in the section on material and methods were taken, the successive readings of single manometers proved to be quite regular regardless of the stage of development of the contained egg or young turtle. Examples of the actual oxygen consumption during individual experiments taken at random from our protocols are shown in Figure 2 to illustrate the various lengths of experimental periods, the various times between readings and the obvious regularity of the oxygen consumption.

The mean values of all our experiments on the oxygen consumption of the turtle egg are presented in Table I. The number of individual determinations at each stage varied somewhat but most of the figures in this table are averages of from three to six determinations. These same data are shown graphically in Figures 3 and 4. The figures for the Mud Turtle, Painted Turtle and Three-toed

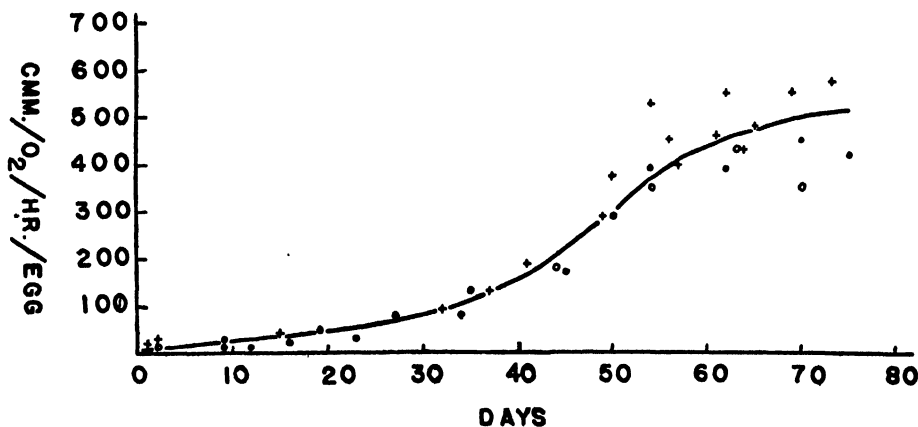


FIGURE 3. Oxygen consumption of the developing eggs of the Mud Turtle, the Painted Turtle and the Three-toed Box Turtle during the period of incubation. ● = Mud Turtle, + = Painted Turtle, ○ = Three-toed Box Turtle.

Box Turtle were fairly close, if identical ages are compared, and they fit rather well to one curve (Fig. 3). The egg of the Snapping Turtle, as was to be expected in view of its greater size, proved to have a much higher oxygen consumption than did the eggs of the other three species (Fig. 4). It has been pointed out that the weight of a newly hatched Painted Turtle is only about 60 per cent of that of a Snapper hatchling and it may be assumed that such weight differences exist even at early embryonic stages. Despite the fact that the curve for the Snapper is at a higher level than that for the other three species, it is obvious that the fundamental nature of the curve is the same in all the forms investigated. Its general shape corresponds to that of the respiratory curves described for the hen's egg (Bohr

and Hasselbalch 1900, 1903; Murray 1925). It should be noted that the curve for the oxygen consumption of the Snapper egg shows a somewhat steeper slope during the period between the thirtieth and fiftieth days than does the curve for the other three species. Reference to Figure 1 shows that this must be related to the more rapid increase in size shown by the Snapper embryo during this time. The similarity of the curves for oxygen consumption to those for length increase is quite striking.

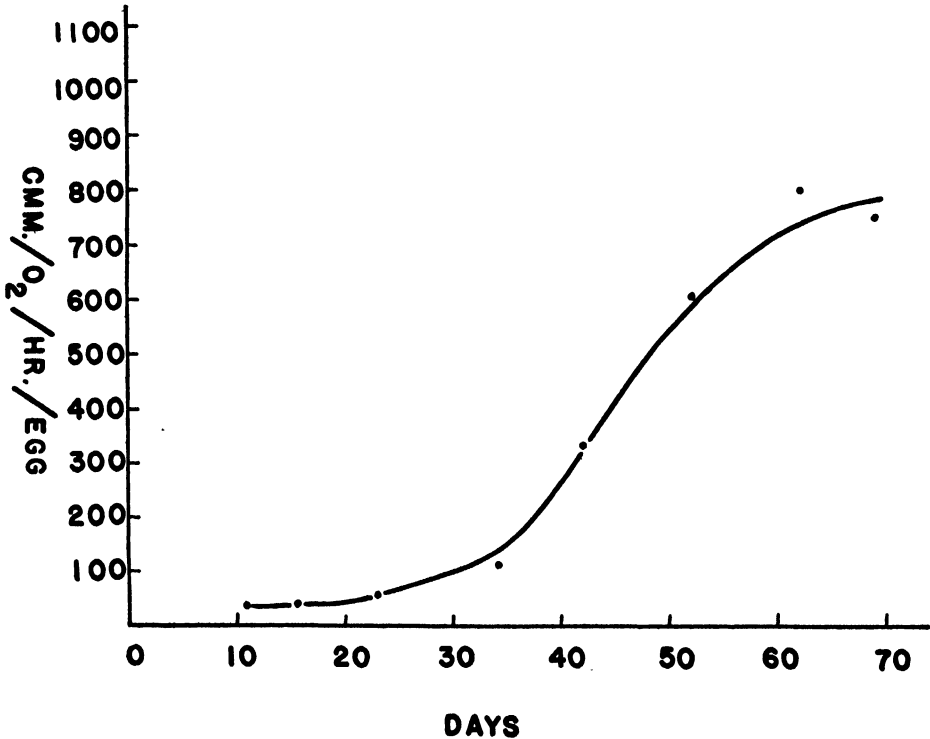


FIGURE 4. Oxygen consumption of the developing eggs of the Snapping Turtle during the period of incubation.

In the case of the turtle one may say that, in general, during the first 50 days of incubation the oxygen consumption is a little less than doubled every 10 days. The single points deviate from the curve but slightly during the first 40 to 50 days but later the deviations become quite pronounced. This period of irregularity corresponds more or less with the period when the curve flattens out. There is, of course, no doubt that the movements of the embryo increase during the latter part of development and it can be assumed that variations in muscular activity are responsible for the variability in the rates of oxygen consumption of older embryos. That this assumption is correct can be inferred from another observation. In Table I are listed some values for turtles that were in the process of hatching and it will be seen that these values are higher than both those for advanced embryos and those for young hatched specimens. The obvious explanation is that

the breaking of the egg shell and emergence from the egg require special muscular efforts and so necessitate a higher oxygen consumption during that time.

It should be noted that the values listed in Table I for the oxygen consumption of hatched turtles are about the same as, or only slightly higher than, those for advanced embryos. This is in line with the observation of Zarrow and Pomerat (1937) on embryos and young of the Green Snake. In the case of the chick however the young, during the first ten days, consumes about five times as much oxygen per hour as does the embryo just before hatching (Lussana 1905). This is doubtless to be ascribed to the great amount of muscular activity of the young chick.

The average oxygen consumption per gram per hour for the hatched turtles were as follows: Mud Turtle, 178 cmm.; Painted Turtle, 186 cmm.; Snapping Turtle, 106 cmm. An exact comparison with rates of oxygen consumption of adult turtles is not possible since no data are available from experiments performed

TABLE II

*Individual variations in the oxygen consumption of eggs of the same age*

| Species               | Age in days | O <sub>2</sub> consumption in cmm./egg/hour |     |     |     |     |     |
|-----------------------|-------------|---|-----|-----|-----|-----|-----|
|                       |             |   |     |     |     |     |     |
| Snapping Turtle       | 24          | 59  | 67  | 39  | 52  | 59  |     |
| Snapping Turtle       | 52          | 806   | 355 | 624 | 592 | 710 | 516 |
| Snapping Turtle       | 62          | 730   | 914 | 553 | 860 | 884 | 845 |
| Three-toed Box Turtle | 27          | 82  | 73  | 85  |     |     |     |
| Three-toed Box Turtle | 44          | 206   | 153 |     |     |     |     |
| Painted Turtle        | 37          | 140   | 144 | 116 |     |     |     |
| Painted Turtle        | 54          | 493   | 538 | 494 | 565 | 536 |     |
| Painted Turtle        | 76          | 645   | 568 | 636 | 581 |     |     |

near 25° C. Baldwin's (1926) figures for experiments conducted at 20° C. are: Painted Turtle (*Chrysemys picta marginata*) of 1,200 gm., 49.2 cmm./gm./hr.; Snapping Turtle of 1,700 gm., 56.2 cmm./gm./hr. If, as can be supposed, the temperature coefficient of the turtle respiration is in the normal range of about 2, the oxygen consumption per unit weight of young specimens is somewhat higher than that of adults. This would be in agreement with the findings of many investigators for both cold-blooded and warm-blooded animals. In view of the uncertainty of the temperature coefficient it seems premature to try to calculate whether identical values for juvenile and adult specimens would be obtained if the calculations were based on surface area rather than on weight.

In connection with the curves shown in Figures 3 and 4 it is necessary to emphasize that the individual points on the curves represent averages of several determinations and that, in some cases, there were considerable differences in the rate of oxygen consumption of different eggs of the same age. The extent of such variations is indicated by some examples presented in Table II. In most cases we are not able to account for these variations but it is clear that not all eggs developed at exactly the same rate in spite of the uniformity of the external conditions and therefore we occasionally found that two eggs of the same lot, opened on the same day contained embryos which differed significantly in size. For example, two Painted Turtle eggs taken from the same female and incubated for 50 days

both contained living and seemingly normal embryos but one embryo measured 13.0 mm. while the other measured 16.5 mm. Similar cases could be cited for the Snapper and the Three-toed Box Turtle. At the later stages of development it is probable that individual differences in the amount of movement of the embryos during the period of the experiment would suffice to account for most of the variations in oxygen consumption. It is, in fact, apparent from our protocols that, during the late stages, a single egg used on successive days often showed a regular, but relatively high rate of oxygen consumption on one day and a regular but low rate on another. There would thus seem to be some periods of activity of fairly long duration and other periods when the embryo is quiescent.

Despite the individual variations discussed above it is clear, from examination of Figures 3 and 4, that the number of determinations and the number of eggs used in these experiments must have been large enough to eliminate any great errors due to variations. Otherwise one would hardly expect such smooth curves.

#### *Changes in weight, dry substance and fat content during development*

Previous observations on weight changes in the eggs of turtles have indicated that there is a considerable water intake during development. Cunningham and Hurwitz (1936) report a 37 per cent increase in the weight of the egg of the Diamond-back Terrapin and a 50 per cent increase in the egg of the Marine turtle, *Caretta caretta*. Cunningham and Huene (1938) found that the Snapping Turtle egg may show a maximum weight increase of 60 per cent and the Painted Turtle egg a maximum increase of 75 per cent.

In view of these results Needham (1942) lists the chelonian egg as a non-cleidoic egg, one which is dependent upon uptake of water from the environment for successful incubation. Although this subject was not of immediate concern in the present investigation, some of the eggs of each species were weighed from time to time and the results seem worthy of mention as indicating that the eggs of different species are quite differently constituted in respect to their water requirements and that the amount of water intake necessary for successful development need not be so great as the figures of Cunningham and Huene would indicate. In our experiments the eggs of the Snapping Turtle, the Painted Turtle and the Three-toed Box Turtle all showed an increase in weight during development but the increases were not so great as those reported by Cunningham and Huene. This difference is probably due to a difference in conditions. All of the eggs in our experimental series were kept on cotton which was constantly kept moist and the incubator always had some moisture condensed on the walls. Under these conditions a high percentage of the eggs hatched successfully. On the other hand, eggs kept on moist cotton on a laboratory shelf lost water rapidly, as evidenced by collapsing of the shells, and it was found necessary to keep these eggs covered by a layer of wet cotton in order to bring them through to hatching. The eggs used by Cunningham and Huene were buried in moist sand.

A group of eight Painted Turtle eggs which were kept in the incubator were weighed at approximately weekly intervals throughout their development and the young turtles from these eggs were weighed shortly after hatching. The initial weights, taken within 2 hours after removal from the oviducts, ranged from 4.858 gm. to 5.773 gm. and gave an average of 5.275 gm.; at 60 days incubation the weights ranged from 5.821 gm. to 6.349 gm. and averaged 6.069 gm. There was

thus a weight increase of 0.794 gm., approximately 15.1 per cent of the initial weight. It is of interest that this increase was made mostly during the first 9 days of development and that after that time the weights of all eggs stayed fairly constant. The average weight of these eggs 24 hours after removal from the oviducts was 5.286 gm. but at the next weighing (9 days) the average weight was 6.020 gm. The young which hatched from these eggs varied in weight from 3.867 gm. to 4.470 gm. and averaged 4.205 gm.

Our data for the Snapping Turtle and the Three-toed Box Turtle, although not so complete as those for the Painted Turtle, indicate a similar water uptake from the environment. The average weight of ten Snapping Turtle eggs at one day of incubation was 9.944 gm.; at 61 days they had increased in weight to give an average of 10.979 gm., an increase of 10.4 per cent. The average weight of five Three-toed Box Turtle eggs was 8.153 gm. at one day and 9.66 gm. at 70 days, an increase of 18.5 per cent.

Only four eggs of the Mud Turtle were available but these, although they were kept in the same dishes as the Painted Turtle eggs and were weighed at the same times, gave strikingly different results. The four eggs had initial weights of 4.010 to 4.370 gm. and averaged 4.211 grams. The averages for the later weighings were as follows: 9 days, 4.120 gm.; 19 days, 4.144 gm.; 28 days, 4.106 gm.; 38 days, 4.147 gm.; 54 days, 4.120 gm.; 60 days, 4.112 gm. Thus these eggs under the same conditions as those of the Painted Turtle showed no significant change in weight during the whole developmental period. The difference between these two is probably to be attributed to the difference in the nature of the egg shell for the shell of the Mud Turtle egg is hard, thick and brittle while the Painted Turtle egg has a shell which though tough, is more or less parchment-like and can be dented with the finger without breaking. The eggs of the Snapper and Three-toed Turtle have thicker and more rigid shells than do those of the Painted Turtle but do not have the brittle, china-like appearance of Mud Turtle eggs.

In view of the fact that a high percentage of the eggs in our experimental series hatched and gave normal young turtles many of which are still alive we may conclude that the egg of the Mud Turtle is a cleidoic egg in Needham's sense, requiring no uptake of water for its successful development and that the eggs of the other three species used are able to carry through their development with much less water uptake than was previously believed. Just what the minimum requirements may be remains to be investigated.

The initial differences in weight of the eggs of the different species used are, of course, chiefly due to characteristic differences in size of the eggs. In the extensive literature dealing with the natural history of turtles it is customary to indicate these size differences by giving measurements of the length and breadth of the egg but since turtle eggs are often rather irregular in shape we feel that the volume of the egg furnishes a more reliable index of its relative size. The average volumes of the eggs used in the present work were as follows: Mud Turtle, 3.7 cc.; Painted Turtle, 5.3 cc.; Three-toed Box Turtle, 8.1 cc.; Snapping Turtle, 8.7 cc. The limits of individual variation were: Mud Turtle (4 eggs), 3.6-4.0 cc.; Painted Turtle (26 eggs), 4.7-6.1 cc.; Three-toed Box Turtle (12 eggs), 7.1-9.0 cc.; Snapping Turtle (17 eggs), 7.5-9.9 cc. It may be of interest to note that in our largest clutch of Snapping Turtle eggs (61 eggs) there were two which were unusually large, having volumes of 15.3 and 13.5 cc. respectively. These when

opened proved to be double-yolked eggs both of which were, unfortunately, infertile. We have seen no other double-yolked turtle eggs nor have we encountered any reference to them in the literature.

Our data on the changes in dry substance and in fat content apply to the Snapping Turtle only and were derived from determinations on 20 eggs (at 15 days incubation) and on 17 newly hatched turtles. The eggs were analyzed in two batches of ten eggs each and the turtles in two batches of ten and seven animals respectively. The results of these determinations are summarized in Table III.

TABLE III

Changes in dry substance, water content and ether extract during the development of the Snapping Turtle egg. The values are expressed in grams per egg (or, in the case of hatched turtles, in grams per individual) and are mean values of two determinations. The single values are given in parentheses.

|               | Egg contents            | Egg shell            | Hatched turtles         | Decrease during development |
|---------------|-------------------------|----------------------|-------------------------|-----------------------------|
| Fresh weight  | 8.80<br>(8.54, 9.05)    | 1.56<br>(1.56, 1.57) | 7.36<br>(7.54, 7.18)    | 1.44                        |
| Dry weight    | 1.91<br>(1.89, 1.92)    | 1.14<br>(1.15, 1.13) | 1.45<br>(1.52, 1.38)    | 0.46                        |
| Water         | 6.89<br>(6.65, 7.13)    | 0.42<br>(0.41, 0.44) | 5.91<br>(6.02, 5.80)    | 0.98                        |
| Ether extract | 0.273<br>(0.258, 0.288) |                      | 0.212<br>(0.220, 0.203) | 0.061                       |

These data show that the hatched animal has considerably less water than the original egg and about 0.5 gram less dry substance. Much of the water loss is to be accounted for by the fluids of the amniotic and allantoic cavities which are lost at hatching. We have no precise measurements on this for the Snapping Turtle but one of the Mud Turtle eggs was opened on the 77th day and the following weights were obtained: whole egg, 4.341 grams; embryo, 3.207 grams; shell, 0.674 gram; fluid, 0.409 gram; unaccounted for, 0.054 gram. The fluid lost at hatching thus amounted to at least 9.4 per cent of the weight of the whole egg just before hatching. A comparable percentage is undoubtedly lost in the Snapping Turtle. Although no determinations of the inorganic substances were made, it can be assumed that the loss of dry substance was primarily due to the oxidation of organic material.

Fat was consumed to the extent of 61 mg. per egg. Since one gm. of fat of average composition requires 2,020 cc. of oxygen for total oxidation the fat disappearing from the Snapper egg would require 123 cc. of oxygen for its combustion. The total oxygen consumption during development up to hatching, as computed from Figure 4, is about 500 cc. This leaves about 380 cc. of oxygen for oxidation of the 400 mgm. of dry substance additionally consumed (total dry substance used, minus fat consumed). Four hundred mgm. of carbohydrate are totally oxidized by 300 cc. of oxygen and the same amount of protein by 400 cc. of oxygen. It

would appear possible, therefore, that relatively large amounts of protein have been consumed. In view, however, of the fact that relatively small errors in the fat figures might shift the emphasis towards the carbohydrates (if instead of 61 mgm., 100 mgm. of fat had disappeared, then about 300 cc. of oxygen would be left for residual oxidations), more definite conclusions will have to wait for direct determinations of carbohydrates and proteins or for determinations of the respiratory quotient. Nevertheless, the evidence available from other sources does support the idea that perhaps large amounts of protein are catabolized during the development of turtles. Karashima (1929) observed that the egg of *Thalassochelys corticata* loses 0.52 gm. of fatty acids up to the time of hatching while Nakamura (1929) found a loss of 0.086 gm. of nitrogen during the first 45 days of development. This figure for nitrogen loss corresponds to an approximate protein degradation of 0.53 gm. per egg. It does not however represent the entire protein degradation, since no hatched turtles were studied and it may be pointed out that, according to Karashima's report, only 0.28 gram of fat was used, during the first 45 days of development.

#### SUMMARY

1. The average period of development of the Mud Turtle egg at 25.0 to 25.5° C. is 76 days; that of the Painted Turtle egg, 63 days; and that of the Snapping Turtle egg, 72 days.

2. Measurements of the carapace lengths of developing embryos of the Painted and Snapping Turtles indicated a relatively slow growth rate during early development, a rapid rise during the period between the thirtieth and sixtieth days, and almost no increase in length from the sixtieth day up to hatching.

3. The figures for the oxygen consumption of the developing eggs of the Mud Turtle, Painted Turtle and Three-toed Box Turtle agree closely and can be expressed by a single curve while those for the Snapping Turtle egg, though they give a fundamentally similar curve, show a significantly higher rate of metabolism. This is to be attributed to the greater bulk of the Snapper embryo.

4. During the first 50 days of development the oxygen consumption was a little less than doubled every 10 days; later on the curves flatten out.

5. While the increase in oxygen consumption is quite regular during the first 40 to 50 days of development irregular values were obtained at later stages, probably as a result of muscular activity of the embryos.

6. Young hatched turtles have about the same rate of oxygen consumption as do advanced embryos. The highest values for oxygen consumption were obtained from eggs studied while in the process of hatching.

7. The eggs of the Snapping Turtle, Painted Turtle and Three-toed Box Turtle showed a definite water intake during development as evidenced by a progressive weight increase but no such phenomenon was found in the case of the Mud Turtle egg.

8. The Snapping Turtle egg shows an average loss, during development, of 0.46 gram of dry substance. Fat is consumed in the amount of 61 mg. per egg. These data in conjunction with those on the oxygen consumption make it appear possible, though not yet certain, that large amounts of protein are catabolized during the development of this animal.

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# THE DEVELOPMENTAL HISTORY OF AMAROECIUM CONSTELLATUM. 1. EARLY EMBRYONIC DEVELOPMENT

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## INTRODUCTION

The Tunicata have held the interest of biologists since Kowalevsky established their taxonomic position by identifying the larvae with Chordates. In their embryology apparent mosaic development has presented a rich field of investigation for both descriptive and experimental embryology. Conklin's exhaustive study of *Styela* (1905) established the pattern of development which has been verified for *Asciidiella*, *Ciona*, *Molgula*, *Botryllus*, *Phallusia*, and also for *Amphioxus*. These forms agree in having a moderate supply of yolk that neither obscures nor interferes with the pattern of development. Studies on the early embryology of heavily yolked forms have been restricted to papers on *Distaplia* (Davidoff, 1899-01) and *Amaroecium proliferum* (Maurice et Schulgin, 1884). Since these investigations are incomplete it was thought that the study of an egg with abundant yolk might be of interest in analyzing the extent to which yolk modifies the processes of mosaic development. *Amaroecium constellatum* was chosen because of the apparent twisting of symmetry in its axial structures, the neural tube lying to the left of the notochord rather than dorsal to it.<sup>1</sup>

## MATERIAL AND METHODS

*Amaroecium constellatum* is a compound Ascidian commonly known along the eastern shore of the United States as "sea pork." The zooids are elongate and clustered together to form thick fleshy colonies. The gonads are located in the long post-abdomen, testes posterior to the ovaries which crowd up against the lower part of the abdomen. Fertilization is internal and the embryos develop within "brood spaces." The embryos are located along the length of the ascidiozooid according to the degree of development, the eggs and early stages being lodged in the post-abdomen and lower abdomen; the later stages of tadpoles, in the thoracic region from which they escape when development is completed. The shape of the eggs varies by crowding from spherical to polyhedral. The average diameter of the fixed egg is 250 micra.

The breeding season extends throughout the summer months. All stages are abundant during July and August. During the latter part of June and the early part of September the embryos are few in number. Eggs and embryos are obtained by squeezing the adult colonies in a finger bowl of sea water. Some of the

<sup>1</sup> I express deep gratitude to Professor E. G. Conklin for his interest and encouragement in the preparation of this paper.

earlier stages may be obtained by dissecting the individual members of colonies under a low power microscope. The post-gastrulation stages will continue to develop in tanks in the laboratory but the pre-gastrulation stages are extremely sensitive. They disintegrate shortly after their removal from the adults without completing the divisions then in progress. These embryos must be used as soon as removed and when the material is fresh.

The study of whole embryos is made difficult but not impossible by the character and arrangement of the test cells. Observations, as far as the establishment of the neural plate, were made on whole specimens. They were fixed in Bouin's fluid and preserved in 70 per cent alcohol in which they were mounted on shallow depression slides in vaseline cells to permit rolling them about for examination. The picric acid is retained strongly by the yolk granules whereas the yolk-free cytoplasm, immediately about the nucleus, is colorless. The contrast provides a reliable means for the identification of cells. The position of the spindles can be ascertained and the orientation of the cells known with certainty. Both reflected and transmitted light were used.

Berrill's (1932) technique of hydrolyzing the test with digestive juices was tried but by the time the closely applied test was removable the enzymes had attacked the cells themselves.

The critical stages immediately preceding and following gastrulation were drawn with the aid of a camera lucida, then embedded and sectioned in the manner suggested by Doctor Eleanor Slifer (oral communication) for yolk-laden eggs. All reconstructions were made from serial sections studied in conjunction with whole cleared specimens.

This study deals with the embryo as far as the end of gastrulation. A second paper is concerned with organogenesis in the tadpole.

#### EARLY DEVELOPMENT

Since there are many maturation spindles in evidence in the unfertilized eggs but no polar bodies formed, it seems reasonable to conclude that *Amaroecium* agrees with *Styela* in extruding its first polar body at fertilization. The egg at this time is plentifully supplied with yolk granules. The test cells are embedded in the peripheral cytoplasm in a compact layer and the follicle cells are tightly pressed against the chorion. After fertilization the test cells are clustered outside the egg and inside the chorion in small irregular groups, some remaining embedded in the cortical cytoplasm. The chorion is lifted from the surface leaving a wide perivitelline space. As cleavage proceeds the test cells completely fill up this space as well as any available cleavage furrows and depressions on the surface of the young embryo. On its external surface the test presents the appearance of a pavement epithelium. In section it consists of compact layers of spindle shaped cells with deeply staining nuclei.

The first cleavage is meridional and divides the egg into two blastomeres which represent the right and left halves of the future embryo. The right blastomere is slightly smaller than the left one (Fig. 1, C; 2, B). In some cases the disparity seems greater than in others, due, probably, to variations in shape of the egg resulting from pressure of contiguous eggs. It has been shown in the simple *Tunicates* and in *Amphioxus* (Conklin, 1905, 1932) that the cleavage nucleus lies pos-

terior to the center of the egg but in the midline. The disparity in size of the first two blastomeres in *Amaroecium* indicates that the meeting of male and female pronuclei is effected not only in the posterior region of the egg but also to the right side of the midline (Fig. 1, B; 2, A). In *Amaroecium*, therefore, there is a double eccentricity of the zygote nucleus, posterior and to the right of the main axis of the egg.

In all the sections of *Amaroecium* eggs examined the polar bodies lies eccentrically with respect to the main mass of yolk (Fig. 1, A). By assuming that the germinal vesicle always lies to the right of the apex of the elliptical egg and also that the sperm penetrates on the same side it is possible to explain the lateral eccentricity of the zygote nucleus to the right of the median axis; this, in turn, explains the constant inequality in size of the first two blastomeres.

The second division also is meridional and at right angles to the first, thus dividing the egg into two anterior and two posterior blastomeres. The posterior

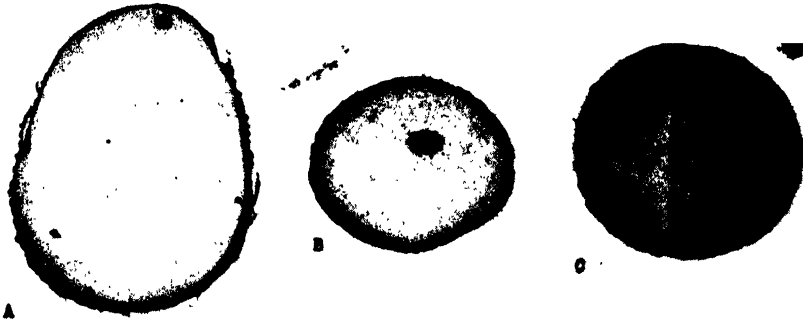


FIGURE 1. Photomicrographs of A. Fertilized egg; first polar body extruded, spindle for second polar body forming. Chorion is broken at the animal pole. Test cells bulge through the chorion. B. Cross section through animal pole of egg after fusion of the pronuclei showing double eccentricity of the nucleus posterior to and lateral to the midplane of the egg. C. Two cell stage; smaller cell is right blastomere. Magnification—250  $\times$ .

blastomeres are smaller than the anterior two. Beginning with the smallest of the four they fall into this order: the right posterior, the right anterior, the left posterior and the left anterior. The nuclei of all lie at the extreme tip of the cells toward the animal pole (Fig. 2, C, D). The third cleavage cleaves the egg in the latitudinal plane into four micromeres at the animal pole and four heavily yolked macromeres at the vegetative pole.

The nuclei and their areas of yolk-free cytoplasm elongate in the direction of the next division and the spindle remnants remain clearly evident after the division is completed. These two features constitute valuable means of identifying the axes of the embryo.

The spindles of the two anterior micromeres are parallel with the antero-posterior axis whereas in the two posterior micromeres they are transverse to this axis and the posterior cells form an arc around the anterior cells (Fig. 3). The elongate cytoplasmic areas of the posterior macromeres in preparation for their next

division converge towards the animal pole. The cytoplasmic areas of the anterior macromeres lie parallel with the lateral borders of the overlying micromeres (Fig. 3).

It will be convenient for purposes of ready reference to designate the cells by the letters used by Conklin in his study of cell lineage in the Tunicates. By underlining the cell designation of the left side he distinguishes them from their corresponding members on the right side. In the eight cell stage the anterior left macromere is A 4.1, the right one is A 4.1. The corresponding micromeres are

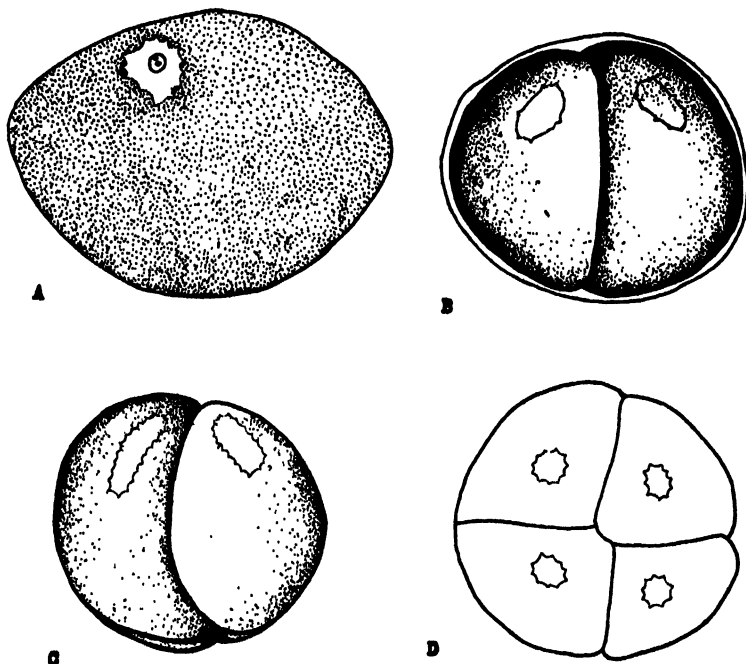


FIGURE 2. A. Fertilized egg before first division, B. Two cell stage; posterior view, C. Four cell stage; anterior view, D. Polar view of four cell stage. Vegetative pole position of nuclei showing. Except where noted all magnifications are 266  $\times$ .

a 4.2 and a 4.2. The posterior left and right macromeres are B 4.1 and B 4.1 respectively. Their corresponding micromeres are b 4.2 and b 4.2 (Fig. 3).

The fourth division is significant in that it accomplishes the distribution of cytoplasmic substances to areas similar to those recognizable in forms with less yolk in their eggs. All the micromeres divide at approximately the same time. Both pairs of macromeres divide unequally into a smaller pair of cells toward the animal pole and a large pair of yolk-charged macromeres occupying the entire vegetative hemisphere. The median derivatives of the anterior pair constitute the chorda-neural crescent, the posterior derivatives are combined mesoderm and ectoderm. All sixteen cells can be seen in polar view (Fig. 4).

Since the cleavages are not synchronous beyond the sixteen cell stage it may be

well at this point to summarize the cell lineage in the egg of *Amaroecium*. The micromeres, designated by small letters in the figures, produce the ectodermal cells that eventually grow over all the other cells of the embryo. The "A" macromeres give rise to the chorda-neural crescent, A 5.2 and A 5.2, and half of the endodermal quadrant, A 5.1 and A 5.1. The "B" macromeres give rise to the mesodermal crescent, B 5.2 and B 5.2, and the other half of the endodermal quadrant, B 5.1 and B 5.1 (Fig. 4).

The pattern corresponds exactly to the pattern of Ascidian mosaic development in *Styela*. The generous provision of yolk in the egg of *Amaroecium* prevents the appearance of a blastocoele cavity and disposes the presumptive mesodermal cells to a position farther towards the animal pole than this crescent of cells occupies in the egg of *Styela* where the presumptive endoderm cells are much smaller in size. The hereditary pattern of development is not disturbed by the accumulation of yolk. The mechanics of the process are altered but the same relative positions of the presumptive embryonic areas are assumed. The embryonic areas consist of an endo-

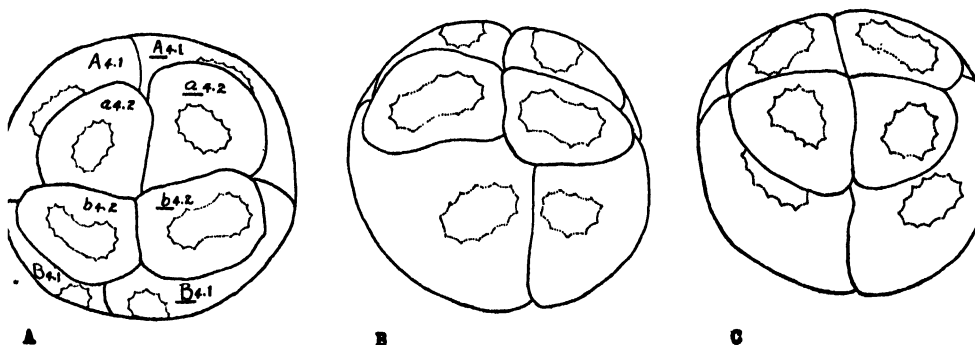


FIGURE 3. Eight cell stage; direction of spindles indicated by stippled areas, A. Animal pole, B. Posterior view, C. Anterior view.

dermal quadrant of large macromeres on the dorsal side, i.e., toward the vegetative pole, with a chorda-neural crescent of two cells lying anterior to them and a mesodermal crescent of two cells lying posterior to them. The ectodermal micromeres occupy the entire animal pole or future ventral side of the embryo. All the cells on the right side are smaller than their sister cells on the left side. Otherwise the cells are disposed symmetrically with respect to the median plane of the embryo.

Before the fifth cleavage the micromeres shift in position; the "a" cells spreading transversely, b 5.4 adjacent to them and b 5.3 overlapping the mesodermal cells (Fig. 5, A). The embryo passes through a twenty-two cell stage in its fifth cleavage. The macromeres and their derivatives divide first, those on the right side preceding those on the left. The mesodermal cells divide meridionally, increasing the number of cells in that arc to four. The posterior macromeres also divide meridionally but unequally, producing two smaller lateral mesodermal cells towards the animal pole and two median elongate macromeres (Fig. 5, C, E). Each of the anterior pair of endodermal macromeres divides into two unequal cells, a smaller one on each side of the chorda-neural crescent and a larger median one (Fig. 5, D).

The four large macromeres meet in a median furrow at the vegetative pole. The chorda-neural cells divide later into a transverse row of four cells.

In the twenty-two cell stage there are three mesodermal cells on the right side, B 6.2, B 6.3, B 6.4 and their corresponding cells on the left (Fig. 5, 6). When they divide at the sixth cleavage the dorsal derivative of B 6.2 which is B 7.4 and of B 6.4 which is B 7.8 are presumptive muscle cells. The ventral members and both derivatives of B 6.3 are mesenchyme. Reference to Figure 5 will show that B 6.3 lies at the mid-region of the posterior lip of the blastopore. At this division the

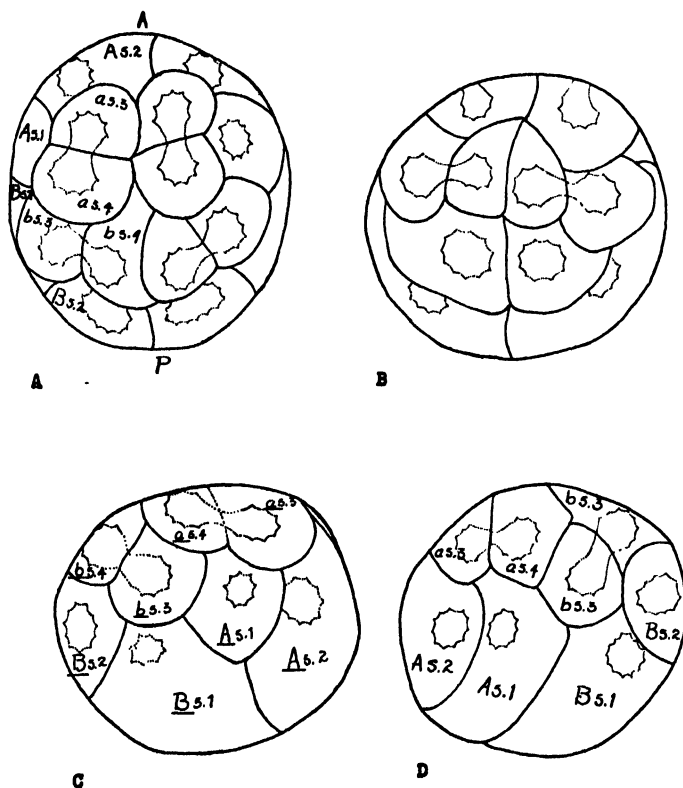


FIGURE 4. Sixteen cell stage, A. Animal pole, cells on right side designated, B. Posterior view, C. Left side, D. Right side.

chorda-neural cells divide into two transverse rows of four neural cells and four chordal cells. The chordal cells lie towards the vegetative pole, the neural cells towards the animal pole and in contact with the ectodermal micromeres (Fig. 7).

No attempt is made beyond this point to follow the lineage of the cells. Since they correspond through the first six cleavages with the cells of Ascidians whose cell lineage can be followed through gastrulation, it may be assumed that their agreement continues through subsequent stages with differences dependent on the mechanics of gastrulation in Amaroecium.

It may be helpful before presenting the process of gastrulation to clarify the terms used in describing it. The blastopore is the margin of cells surrounding the macromeres. Its anterior border consists, at first, of chorda-neural cells and later of neural cells only. It is consistently called the anterior lip (the "dorsal lip" of embryologists dealing with Amphibian forms). Its posterior border ("ventral lip") is the crescent of presumptive mesodermal cells and is called the posterior lip. Lateral regions are referred to as right and left lips respectively.

In *Styela*, which provides the pattern to be used as a basis for comparison, movements of cells in gastrulation are not modified by accumulation of yolk. The

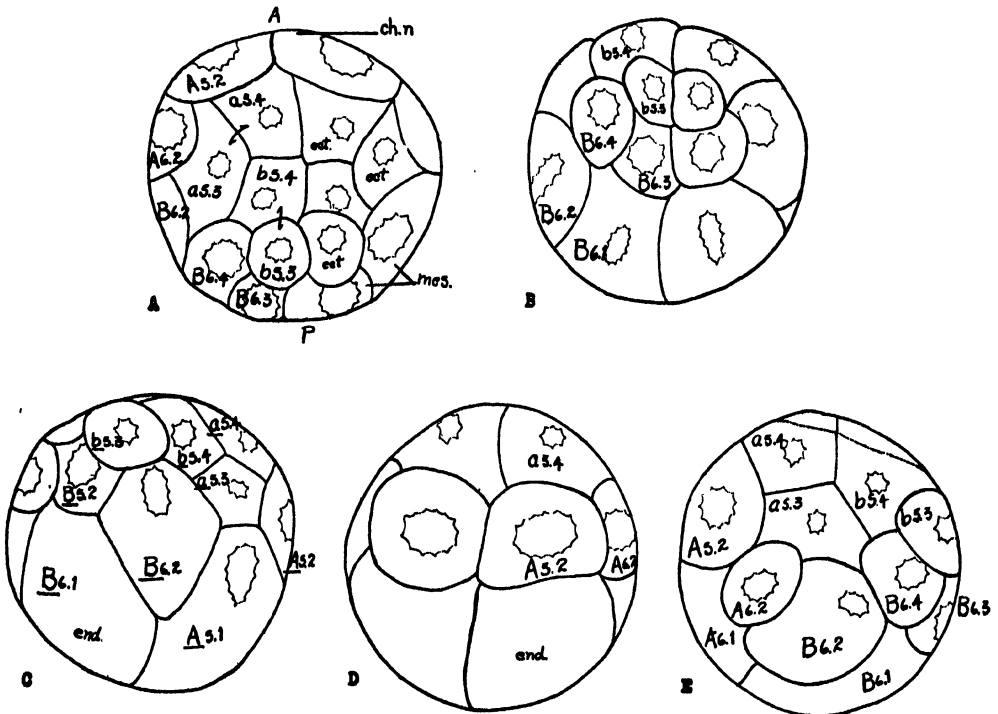


FIGURE 5. Twenty-two cell stage stage.

A. Animal pole; *ch. n.*—chorda-neural crescent; *mes.*—mesodermal crescent; *ect.*—ectodermal micromeres, B. Posterior view, C. Left side, D. Anterior view, E. Right side.

first cells of the mesodermal crescent to be inturned at the lateral lips of the blastopore are mesenchyme cells which come to lie ventrally in the trunk region of the embryo. The cells that converge medially to form the lateral lips after the mesenchyme invaginates are presumptive muscle cells of the tail. The final blastopore is T shaped, neural cells forming the anterior lip, muscle cells forming the lateral margins of the posterior lip. The posterior lip forms the limb of the T where the lateral lips converge toward the mid-plane in a groove. At the posterior-most point in the groove the caudal mesenchyme cells are lodged. Chordal and endodermal cells invaginate, the latter forming a typical archenteron. The blasto-

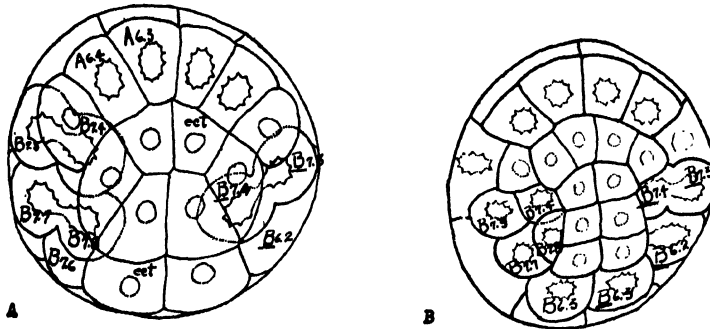


FIGURE 6. A. Thirty-two to sixty-four cell stage; division on the right side in advance of the left side. B. Same stage, mesodermal cells having completed division on the right side but lagging on the left.

pore closes by posterior growth of the anterior lip and growth toward the mid-line of the lateral lips.

The egg of *Amaroecium* departs from this pattern of gastrulation in several respects. The margins of its blastopore are established at the sixth cleavage. The anterior lip consists of four chordal cells, the posterior lip of mesodermal cells. Enclosed by the blastoporal lips are the large vegetative macromeres (Fig.

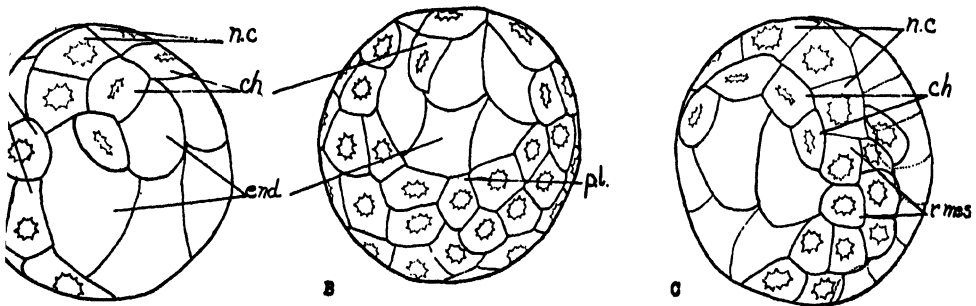


FIGURE 7. Gastrulation.

A. Viewed from the left lateral blastoporal lip, B. The embryo tilted toward the animal pole to show the complete lip of the blastopore. The angle distorts the size of the endodermal area, C. Viewed from the right blastoporal lip; *ch.*—chordal cells; *end.*—endodermal macromeres; *l.mes.*—mesoderm of left lateral lip of blastopore; *n.c.*—neural cells; *r.mes.*—mesoderm of right lip; *p.l.*—posterior lip of blastopore.

7). In subsequent cleavages they divide into cells of unequal size. The cells at the animal pole are fairly uniform (Fig. 5, 6). All the animal micromeres may be called the epiblast. They differentiate into ectoderm which spreads over the embryo by the process of epiboly.

The mass of inert yolk in the macromeres prevents an invagination of the potential endodermal cells. These cells, therefore, do not participate in the early movements of gastrulation. Gastrulation commences with activity of the cells

in the mesodermal crescent. They divide and move over the surface of the macromeres in the direction of the vegetative pole. The cells on the right side of the crescent precede those of the left side in dividing. Smaller size of the macromeres on the right side and accelerated rate of division of these mesodermal cells effect

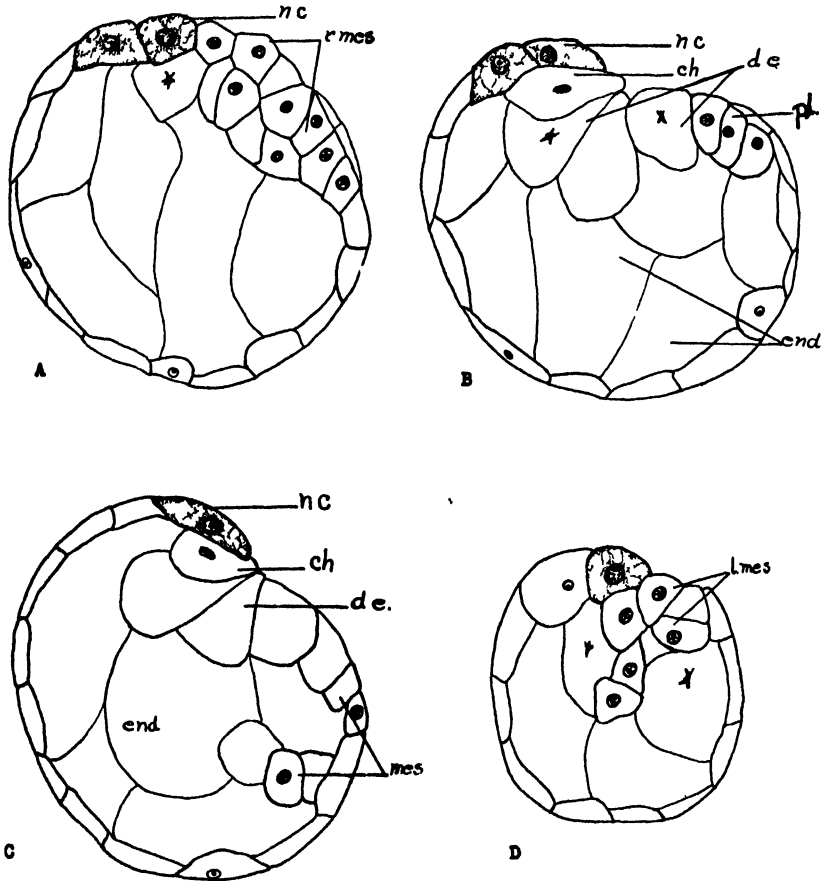


FIGURE 8. Sections through a gastrula during the period of pseudogastrulation.

A. Through the right lip, B Through the middle of the blastoporal lip, C. Through the region immediately to the left of the preceding, showing the mesodermal cells lower on the left side, D. Through the left lip; depression present where mesoderm meets neural cells; *ch.*—chorda; *d.e.*—definitive endoderm or endoderm of the pharyngeal roof; *end.*—yolk-laden endoderm of floor of pharynx; *mes.*—mesoderm.

a change in shape of the blastopore from circular to an irregular oval. More rapid overgrowth of the right lateral region of the posterior margin results in a narrowing of the blastoporal rim on that side in the antero-posterior direction (Fig. 7). The cells converge medially to form lateral lips as they do in *Styela* but they converge more rapidly from right to left and thus distort the shape of the

blastopore. The right lateral margin defines more of a horizontal curve than the left (Fig. 9, A, B). The cells of both chordal and neural crescents, the anterior lip, increase in number to eight.

As the blastopore becomes smaller changes occur in the endodermal area. The macromeres divide into a number of polyhedral cells, those at the surface of the region enclosed by the blastopore being smaller than those constituting the internal yolk mass (Fig. 8, B, C). This layer of cells may be called the endodermal plate. Corresponding in shape with the blastopore it is a small oval region tapering to a narrow point on the anterior right side where the blastoporal lips are approaching each other more rapidly than they are on the left side. The cells of the endodermal plate change in shape from polyhedral to pyramidal, their apices tapering into a cleft-like depression on the surface formed simultaneously with their

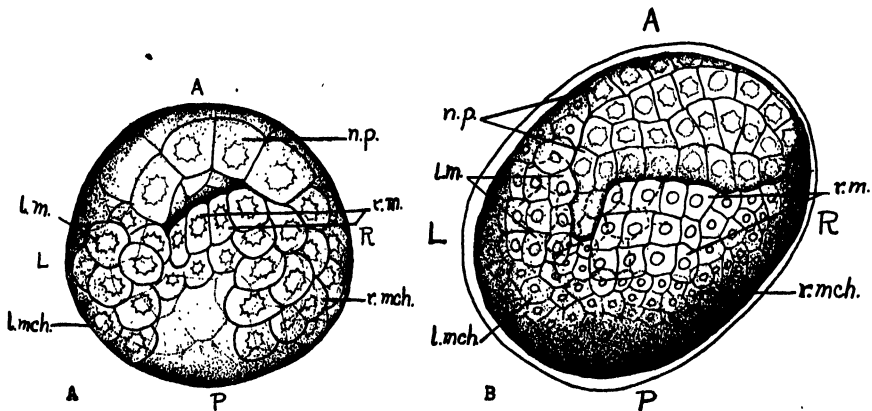


FIGURE 9. Late gastrulation.

A. Dorsal view. Blastopore closed on right side when depression appears in endoderm. Mesoderm growing from right to left in lip of blastopore. B. Posterior view of embryo at the end of the gastrulation period, tilted slightly to show complete left side. Cells with interrupted outlines represent ectoderm, shown only where blastopore has closed; *l.m.*—left muscle cells; *l.mch.*—left mesenchyme; *n.p.*—neural plate; *r.m.*—right muscle cells; *r.mch.*—right mesenchyme.

change in shape. Their broad bases rest on the larger endodermal cells in the interior of the embryo (Fig. 8, B, C). Such an invagination may be called a "pseudo-invagination" since it closes again without the formation of an archenteron.

The chordal cells adjacent to the endodermal plate invaginate with the endodermal cells. They are involuted at the anterior lip of the blastopore and come to lie immediately underneath the neural plate and dorsal to the endoderm (Fig. 8, A, B, C). With involution of the chordal cells the blastopore closes on the right side. The mesodermal cells of the posterior margin on this side now occupy their internal embryonic position (Fig. 8 A). The ectodermal cells have overgrown them and the ectodermal cells meet the neural cells at the right side of the neural plate. The remaining mesoderm curves around the region of "pseudo-invagination" to the left end of the neural plate or anterior lip where the mesoderm has

been proliferating more slowly. The lateral margins of the posterior blastoporal lip are potential muscle cells of the tail; the central region is caudal mesenchyme.

The relationship of the blastopore regions are the same as they are in gastrulation stages of *Styela* where the blastopore is finally T shaped. Convergence of the lateral margins toward the median axial plane is asymmetrical and the lateral margins fuse to the left of the mid-line. The blastopore of *Amaroecium* may be described as an irregular T the right horizontal bar of which is longer than the left (Fig. 9, A, B).

Closing of the blastopore on the right side produces a slight horizontal curve in the neural plate. When the depression in the endodermal plate closes, the cells of the neural plate extend posteriorly. The neural cells slope gradually, the left side lying more posteriorly than the right side (Fig. 10).

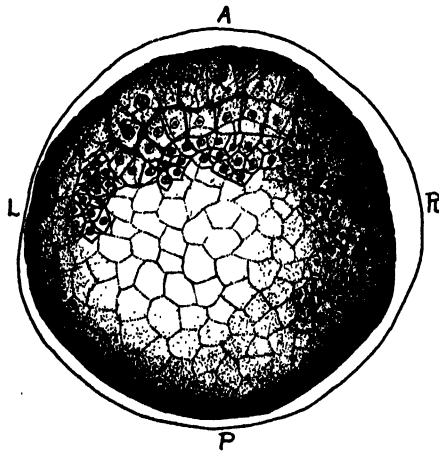


FIGURE 10. Embryo at end of gastrulation. Posterior row of neural cells showing drawn toward mesoderm on left side. Ectoderm in stippled lines; mesenchyme small cells in masses on right and left sides; presumptive muscles stretched across lip and to left of lip.

The activities of all marginal cells in closing the blastopore correspond to the same activities in other Tunicates but the difference in rhythm of division between the cells on the right and the cells on the left deflects the posterior margin of the blastopore to the left (Fig. 8, D). This asymmetrical growth shifts the right muscle cells to the dorsal side of the notochord, the left muscle cells to the ventral side. Convergence and fusion of the cells of the blastoporal margins to the left of the mid-line results in the posterior extension of the neural plate laterally instead of mid-dorsally. The neural tube lies, therefore, to the left of the notochord instead of dorsal to it. *Amaroecium* is the only Tunicate known in which there is a twisting of the neural tube from a position dorsal to the notochord.

When gastrulation is completed the embryo is approximately spherical with a rudiment of a tail. It lacks an archenteron and the posterior region of the neural plate or potential neural tube is asymmetrical, curving through an angle 90° to the left. Except for these two differences it resembles superficially at this early stage the tadpole of other Ascidians.

## DISCUSSION

All the Tunicates whose embryology is known conform to a pattern of mosaic development. *Amaroeicum constellatum* despite the fact that its egg is heavily yolk-laden follows the same pattern with modifications contingent upon the mechanical interference of yolk. As Conklin has pointed out, "Cleavage is less constant and fundamental than the type of localization and the two may be relatively independent."

The ooplasmic substances are distributed to cells that assume the same relationships found in forms not filled with yolk. The main mass of inert yolk remains at the vegetative pole and spreads the early blastoporal lips into a circle wider than that of *Styela* or *Amphioxus* although the cells are disposed in the same pattern. Ectodermal micromeres occupy the animal pole, yolk occupies the vegetative pole, and between these two areas lie an anterior crescent of mesodermal cells constituting the lip of the blastopore.

Three movements concur in carrying the cells to their final positions where they differentiate into the fundamental structures of the adult body, involution or invagination, epiboly, and convergence or the movement of axial structures into their positions in the median plane. Invagination of the endoderm is impossible but what might be considered an abortive attempt at invagination is made in the depression of the definitive endoderm. It may be called "pseudo-invagination." The depression is accomplished by a change in shape of the endodermal cells whereby they are depressed below the surface. It is not an invagination of cells into a segmentation cavity. Neither does it effect the formation of an open archenteron.

As the mesoderm proliferates over the surface of the endoderm the epiblast cells grow over them. By the process of epiboly, therefore, both mesoderm and endoderm are established in their typical relationships. Since the mesoderm continues to spread between ectoderm and endoderm its movement may be regarded as invagination.

Convergence is the process most violently disturbed by the modified pattern of mosaic development. Its disturbance is due primarily to the bilateral inequality in the size of the first two blastomeres which is responsible for difference in rhythm of division between the right and left halves of the embryo. Cell movements of gastrulation, being greater on the right side than on the left, the blastopore becomes asymmetrical in shape. Cells are thus prevented from converging towards the mid-plane. The lateral margins of the posterior border of the blastopore converge and close on the left side and the posterior extension of neural cells curves through a gradual angle to 90° from the mid-dorsal plane.

Dalcq (1938) concludes that the notochord does not induce neural tube formation in the Protochordates as it does in Amphibia (Spemann, 1928). The notochord in *Amaroeicum* is axial and cannot be responsible for the normal asymmetry of the visceral ganglion and neural tube. There is no experimental evidence in the Ascidians to support the dependence of differentiation of the neural tube on presumptive mesodermal tissue. The fact that the asymmetry of the posterior parts of the nervous system in *Amaroeicum* follows the asymmetry of the mesoderm at the blastoporal lip may indicate some degree of dependence between the mesoderm and the differentiation of the nervous system of the trunk in Protochordates.

## SUMMARY

1. The egg of *Amaroecium* contains more yolk than that of any of the other Ascidians whose embryology has been studied.
2. In the two cell stage the right blastomere is always smaller than the left, establishing an inequality in size that persists through subsequent divisions.
3. In the fifth division the cytoplasmic substances are distributed in this fashion: ten ectodermal cells at the animal pole, four endodermal macromeres at the vegetative pole, a crescent of two chorda-neural cells between them on the anterior side, a crescent of six mesodermal cells on the posterior side, two endodermal-mesodermal cells on each side.
4. Decreased size of cells and increased activity in the mesodermal cells on the right side produce asymmetry in the blastoporal lip.
5. Gastrulation is accomplished by the combined processes of overgrowth and invagination.
6. The blastopore closes from right to left, producing a curve in the neural plate through an angle of 90° in the region of the potential neural tube.
7. When the blastopore closes the potential muscle cells lie above and below the notochord, interrupted on the left by the neural tube, on the right by the endodermal rod.

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# PELOMYXA CAROLINENSIS (WILSON) OR CHAOS CHAOS (LINNAEUS)?<sup>1</sup>

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## INTRODUCTION

Considerable confusion exists concerning the name of the large amoeboid organism which was discovered and named *Pelomyxa carolinensis* by Wilson in 1900. The other name by which this organism is known is *Chaos chaos* (Linnaeus). It is the purpose of this paper to show that the valid scientific name is *Pelomyxa carolinensis* Wilson.

## HISTORICAL

The historical data have been presented in full elsewhere (Schaeffer, 1926; Mast and Johnson, 1931) but for clarity of discussion it is necessary to list the pertinent facts.

1. In 1755 Roesel von Rosenhof found an amoeboid organism which he described, figured, and named "der kleine Proteus."

2. In 1758 Linnaeus named Roesel's organism *Volvox chaos* and in 1767 *Chaos chaos* because the name *Volvox* had been used earlier for the colonial flagellate which today bears that name.

3. In 1900 Wilson discovered a large amoeboid rhizopod in North Carolina which he described and named *Pelomyxa carolinensis*.

4. This organism was again found by Penard in France (1902); Kepner and Edwards, in Virginia (1917); Schaeffer, in Tennessee and New Jersey (1937); and Brandwein, Penn, and Shiel, in New York (1943). It is now being maintained in clone cultures by Schaeffer, Belda, Pace,<sup>2</sup> Rice, and perhaps others.

5. Schaeffer (1926) maintains that Roesel's "der kleine Proteus" and Wilson's *Pelomyxa carolinensis* are identical generically and specifically, and that the valid scientific name is therefore *Chaos chaos* (Linnaeus). Stiles (1905), however, believes that the name *Chaos chaos* (Linnaeus) is the valid name for *Amoeba proteus* Leidy, maintaining that Roesel's "kleine Proteus" is like this common laboratory amoeba.

6. Mast and Johnson (1931) present evidence which shows that Roesel's organism "is neither generically nor specifically like either Leidy's *proteus* or Wilson's *carolinensis*." They contend that it is a myxomycete, "an organism usually classified as a plant."

<sup>1</sup> The author desires to express his grateful appreciation to Dr. T. E. Powell, Dr. C. W. Hagquist, and Mr. T. H. Mackintosh of the Carolina Biological Supply Company for many helpful criticisms in the preparation of this paper.

<sup>2</sup> Dr. D. M. Pace, College of Pharmacy, University of Nebraska, kindly furnished the *pelomyxae* from which the author's clone was established.

## FACTS AND DISCUSSION

1. *It is impossible to ascertain the exact structure of Roesel's "der kleine Proteus."* A careful study of Roesel's figures reveals that these are barely more than outlines containing a mass of dots and circles. One could not by any stretch of the imagination consider these figures sufficient basis for the identification of any amoeboid species. What the dots and circles represent is obscure. Schaeffer (1938) states that Roesel knew nothing about nuclei, contractile vacuoles, crystals, etc., since these had not yet been discovered, nevertheless Schaeffer admits that these structures are quite important for identification.

Stiles (1905) believes Roesel's organism is *A. proteus* (Leidy), its valid name being *Chaos diffuens* (Müller); Schaeffer (1926, 1937, 1938) maintains that it is *P. carolinensis* (W.), its valid name being *Chaos chaos* (L.); and Mast and Johnson (1931) contend that it is neither, finding it to be a myxomycete. This difference of opinion is in itself strong support for the contention that reasonable proof of the identity of this organism cannot be found.

The first description of *P. carolinensis* by which it can be identified was published by Wilson (1900). Schaeffer (1926, 1937) states that the organism he found in Tennessee, and the one he found in New Jersey (1937) and now maintains in clone culture, is identical with *P. carolinensis* (W.) and "der kleine Proteus," holding that it should be called *C. chaos* (L.). However, since it is impossible to ascertain the identity of "der kleine Proteus," the priority rule establishes *Pelomyxa carolinensis* Wilson as the valid scientific name.

2. *A comparison of the characters of P. carolinensis (W.) and A. proteus (L.) shows that they are generically distinct.*

Schaeffer (1926, 1937) maintains that *A. proteus* (L.) and *P. carolinensis* (W.) are morphologically quite similar, placing them in the genus *Chaos*. On the basis of serological tests he (1937) suggests that they may be "one and the same species." But he (1916) also finds that *A. proteus* (L.) comprises three distinct species (*proteus*, *discoides*, and *dubia*) which he (1926) later advances to the rank of genera (*Chaos*, *Metachaos*, and *Polychaos*).

This raises two questions. Is there any evidence to show that *A. proteus* (L.) and *P. carolinensis* (W.) are generically identical? Does the evidence justify the creation of three new genera out of the species *proteus*? The second question has been considered elsewhere (Mast and Johnson, 1931). An answer to the first may be found by comparing the chief characters of the two organisms. Reference to Figure 1, a photomicrograph of the organisms in the same microscopic field, clearly shows a great difference in size. Other differences are not so apparent. These are brought out in Table I which summarizes pertinent data from various investigators. Most of the measurements credited to them have been checked by the author without serious disagreement. Nevertheless, it must be borne in mind that all measurements represent averages of numerous determinations on diverse clones.

It will be noted that the two organisms are similar in several respects. Both accomplish locomotion by indeterminate lobopodia and possess bipyramidal crystals, Glanzkörper, and small non-refractile granules. The crystals and Glanzkörper, however, are somewhat larger in *P. carolinensis* (W.) than in *A. proteus* (L.). The table also shows that these organisms differ markedly in size (as

TABLE I  
Summarized comparison of *Amoeba* and *Pelomyxa*

| Character             | <i>Amoeba proteus</i> (Leidy)  | <i>Pelomyxa carolinensis</i> (Wilson)   |
|-----------------------|--|---|
| Length                | Up to 600 $\mu$ (Kudo)   | 1,500–3,000 $\mu$ (Wilson); up to 5,000 $\mu$ (Schaeffer)   |
| Diameter              | 134 $\mu$ (Chalkley)   | 500 $\mu$ (Schaeffer)   |
| Volume                | 0.0024 c. mm. (Chalkley)   | 0.12–1.20 c. mm. (50 to 500 $\times$ 0.0024) (Schaeffer)  |
| Pseudopodia           | Locomotion by indeterminate lobe-podia   | Locomotion by indeterminate lobe-podia  |
| Locomotion            | Comparatively active; recovers from disturbance quickly  | Sluggish; recovers from disturbance slowly  |
| Crystals              | Truncate bipyramidal; up to 4.5 $\mu$ long (Schaeffer); 1.5 $\times$ 2.0 $\mu$ (Rice)  | Truncate bipyramidal; 1.5 $\times$ 2.8 $\mu$ ; up to 10 $\mu$ (Rice)  |
| Glanzkörper           | Up to 4 $\mu$ (Rice)   | Up to 10 $\mu$ (Rice)   |
| Smaller granules      | About 0.7 $\mu$ (Rice)   | About 0.8 $\mu$ (Rice)  |
| Contractile vacu-oles | Typically one at posterior end; systole rapid; new vacuole usually formed at same place; character of permanent organelle (Adolph; Metcalf; Mast); 20–50 $\mu$ ; av. 35 $\mu$ (Rice) | Usually between 5 and 15; as many as 25 or 30; temporary organelle (Belda); systole slow; some gradually disappear (Rice); 30–70 $\mu$ ; av. 36 $\mu$ (Rice)  |
| Nuclei                | Typically uninucleate; discoidal; nucleus and granules easily visible in living specimen; av. dimensions 36 $\times$ 26 $\times$ 18 $\mu$ (Rice)                                     | Typically between 300 and 400 nuclei; up to 1,000 (Schaeffer); ovoidal or discoidal (two strains; Schaeffer); nucleus and granules not easily seen. 16–18 $\mu$ (Wilson); 20 $\times$ 15 $\mu$ (Rice) |
| Food vacuoles         | Variable in size and number; several dozen   | Variable in size and number; up to 100  |
| Reproduction          | Binary fission; nuclear division mitotic   | Tripartite division of mother cell following simultaneous mitotic division of nuclei (Schaeffer)  |

measured by length, diameter, or volume), number and average size of the nuclei, number and character of the contractile vacuoles, and type of reproduction.

These differences are largely those which led Greeff (1874) to establish the genus *Pelomyxa*. He specifically states that the large number of nuclei forms the principal character of the genus. A comparison of the original descriptions of *P. palustris* Greeff (1874), *P. villosa* Leidy (1879), *P. greeffi* Blochmann (1893), and *P. carolinensis* (W.) shows that they are large, sluggish, naked rhizopods containing numerous nuclei, many vacuoles, and large Glanzkörper.

The genus *Pelomyxa*, therefore, includes rhizopods with a multinuclear organization, whereas the genus *Amoeba*, for the most part, those with a uninuclear

# A STUDY OF CHROMOSOME ENDS IN SALIVARY GLAND NUCLEI OF *DROSOPHILA*

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## INTRODUCTION

The exact nature of chromosome ends has remained an unsolved problem in the study of chromosome structure. That the end is a specialized part with a characterized behavior is shown by the fact that an intact chromosome end never enters into a permanent association with any other part of the chromosomes and that its absence causes a change in behavior of the chromosome of which it was a part. This is clearly shown by the behavior in maize of broken chromosomes (McClintock, 1938, 1939, 1941, and 1942). Fusion occurs between the raw ends<sup>3</sup> of the two sister halves of the broken chromosomes resulting in a bridge configuration which breaks at anaphase. This "breakage-fusion-bridge cycle" may continue throughout the endosperm tissues, but in the embryo and plant tissues the raw end becomes "healed" and thenceforth no longer shows any tendency toward fusion. In other words, it then behaves as a normal chromosome end regardless of the type of tissue. This shows that there is a difference between a chromosome with a missing end and one with a "healed" end. The nature of the "healing" (or returning to the normal state) is not understood. Additional evidence that chromosomes are dependent for normal behavior upon certain conditions existing at the end is suggested by the paucity of reports of terminal deficiencies. It would be expected that terminal deficiencies would be found with a frequency, the square of the frequency of intercalary deficiencies requiring two breaks. (A small intercalary deficiency may require only one break by an X-ray, Demerec and Fano, 1941.) Nevertheless, terminal deficiencies occur only rarely and are the exception rather than the rule (Kaufmann, 1939a). This would appear to mean either that they are eliminated (Bauer, Demerec, and Kaufmann, 1938), or that there is a tendency for reunion between the two parts of the chromosome. In either case it is true that chromosomes with missing tips are much more rare than chromosomes with an intercalary region missing. There are enough exceptions, however, to show that it is possible for a chromosome to lose its tip and, in some manner, to reform or heal an end which allows the chromosome to function normally (the

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<sup>3</sup> The term "raw end" is used throughout to denote the new and unhealed end of a tipless chromosome. It carries no connotation concerning the physical and chemical state of the end and is merely the counterpart of "normal" and "healed."

terminal deficiencies reported by Demerec and Hoover, 1936; and Sutton, 1940). Also terminal inversions (Kaufmann, 1936; Kikkawa, 1937 and 1938), although rare, show the ability of a raw end of the chromosome to heal and assume the role of a normal end. From all of these data we get the impression that the end of a chromosome is not so much a separate entity as the word telomere denotes (for a recent discussion, see Muller, 1941) but rather a very special state which can be achieved by other parts of the chromosome under certain conditions. The separation of bivalents at meiosis presents further evidence that the chromosome end is unique in its behavior. Upon complete terminalization of chiasmata the ends of the separating chromatids frequently hold together with an amazing tenacity (for discussion see Hughes-Schrader, 1943a and b). An association of homologous ends may even be achieved without previous terminalization of chiasmata (Schrader, 1940a and b; Hughes-Schrader, 1943a). Also the bouquet stage strongly indicates a special function of chromosome ends, as pointed out most recently by Hughes-Schrader (1943b) and Schrader (1944).

The purpose of the present research has been to select and analyze some phenomenon characteristic of chromosome ends in the hope that the analysis would lead to a more complete understanding of the nature of that part of the chromosome. The phenomenon selected for study was that of association of non-homologous ends. This phenomenon occurs both in salivary gland nuclei of Diptera and in meiotic nuclei of various forms. The present study is limited to the salivary gland chromosomes of two wild type strains of *D. melanogaster* (Oregon-R and Swedish-b).

In salivary gland preparations the distal ends of any of the five long chromosome arms may be found adhered together, usually two-by-two. This phenomenon of non-homologous association of ends has been noted by several workers and its analysis was undertaken by Hinton and Sparrow (1941) and Hinton and Atwood (1941 and 1942). They found that some of the possible combinations of chromosome ends occurred with a significantly higher frequency than did others. The particular combinations of ends which occur with a non-random frequency varied from strain to strain but were characteristic of any one strain.

The present report adds to these conclusions the results from three lines of investigations: one, the study of the physical nature of terminal adhesions as demonstrated by mechanical manipulation; two, the genetical nature of adhesions, and the factors affecting adhesions; and three, the effect on the adhesion pattern of one strain of substituting by genetical manipulation a chromosome or chromosome end from another strain. Since each of these points is best dealt with individually, the discussion of the results is presented in connection with the particular data to which it is pertinent. Therefore, the final discussion is of only a general and summarizing nature.

#### MICROMANIPULATION OF TERMINAL ADHESIONS

For the purpose of studying the physical nature of terminal adhesions a technique was devised whereby two chromosome arms associated by a terminal adhesion could be pulled apart. This was accomplished with the aid of a Chambers micromanipulator, working with partially fixed glands, as suggested by Buck (1942). The glands were dissected from the larvae freehand under a binocular

microscope into a drop of aceto-orcein. At once, before hardening from the fixative resulted, the glands were transferred to a coverslip. A "V" of vaseline on the coverslip prevented the aceto-orcein from flowing away from the glands. The corner of another coverslip was placed over the glands, between the two walls of vaseline, and used to press the glands into a thin layer. The coverslip used to smear the glands was then removed and the coverslip with the smeared gland was inverted and placed on top of a moist chamber on the stage of a microscope above the micromanipulator. The various groups of chromosomes were searched quickly until a terminal adhesion was located. The chromosomes involved were identified and the configuration was drawn, using the 44X objective and 15X oculars. The microneedles were then raised into position and inserted into the chromosomes involved in the adhesion, and pulled apart until the chromosome region between the two needles (including the two adhered ends) broke at some point. This usually involved a marked degree of stretching—from two to three times the original length of the segment. The resulting configuration was then sketched; the figure marked with an ink spot; and the coverslip removed and the tissue further stained with aceto-orcein. The coverslip was subsequently sealed with paraffin face downwards onto a slide. The chromosome configuration was then relocated and the results studied using an oil immersion objective.

Twenty-eight such preparations were successfully completed. In 16 of these, the adhered chromosomes, which were stretched between the microneedles, separated between the two adhered chromosome ends. It should here be emphasized that this did not take the form of a mere separation of temporarily joined chromosome ends, but rather that the chromosome ends were tenaciously held together during the stretching by inter-band-like material which broke only after extreme attenuation. A careful study of the bands on the previously joined chromosome ends failed to reveal a single case in which the ends had gained or lost a band (see Figure 1). The only noticeable effect upon the chromosome ends was, in a few cases, a slight attenuation of the end itself.

Of the remaining 12 preparations, 6 failed to break—the terminal adhesions remained intact even though the chromosomes had been pulled to about three times their original length. After release from the needles, the chromosomes resumed an approximation of their original size and showed no marked effect of the previous stretching. No measurements were made at this point, but the degree of retraction of the chromosomes upon release is probably of the same order as that described by Buck (1942).

Six cases showed intercalary breaks. None of these six breaks were close to the adhered tips, three of them being in the chromocenter and the others in approximately the middle of the chromosome arm (2L in region 28; 3L in 67; and 2R in 52).

Eight successful attempts to disentangle the chromosomes in unbroken nuclei of semi-fixed glands by pulling them free with the microneedles produced three types of configurations. The first type was one in which the chromosomes formed a "star-shaped" configuration with all the bases of the chromosomes joined together and all the tips free. Two such configurations occurred. The second type was a continuous chain. This involved two terminal adhesions (2R-3R; 3L-X) both of which held during the stretching at the expense of the paired bases of the arms.

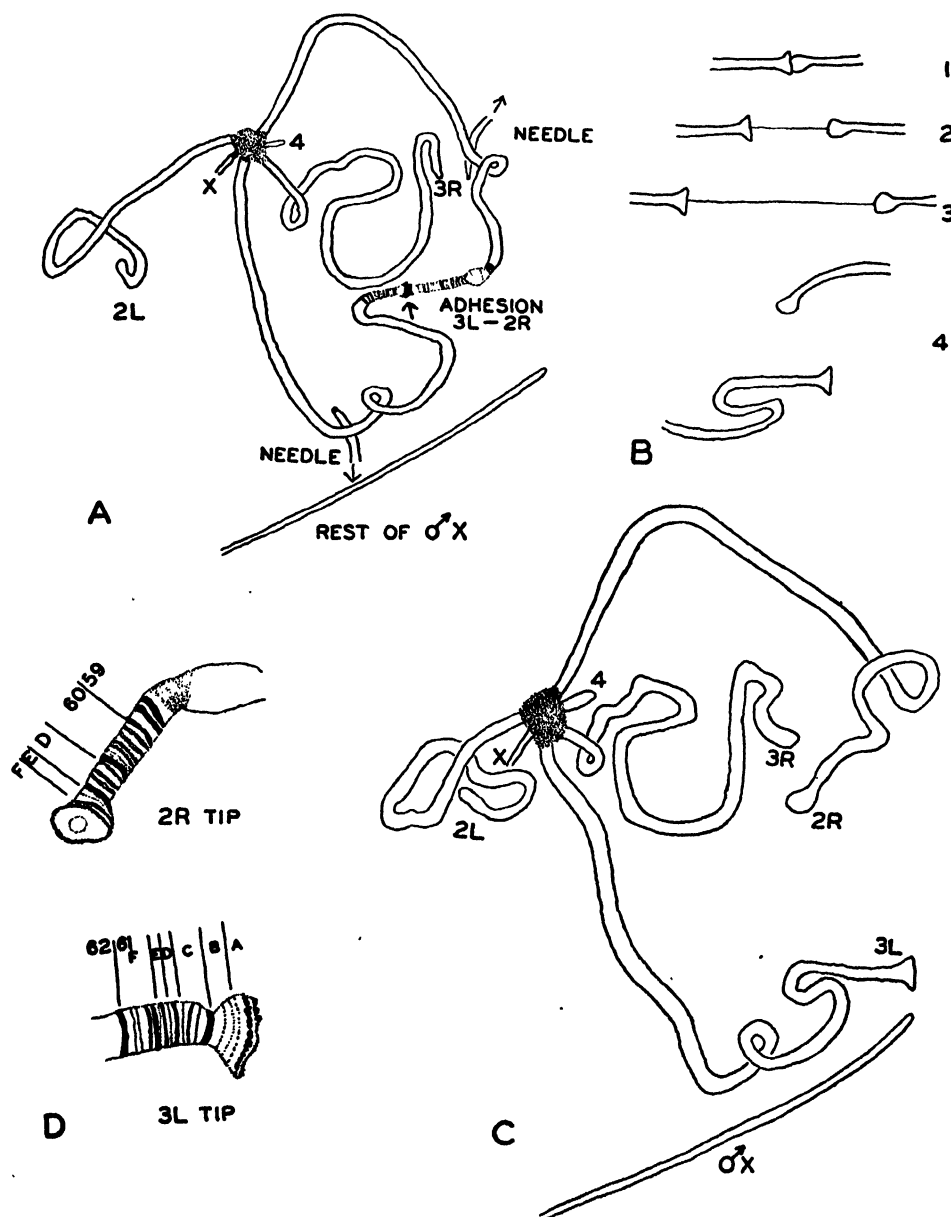


FIGURE 1. *A*. Configuration of chromosomes, sketched with needles of micromanipulator in position. *B*. Behavior of the two adhered ends while the needles were being moved apart. 1. Original position. 2. As the stretching begins a thread-like material pulls out between the two ends. 3. The maximum extent of the stretching of the material between the two tips. 4. The thread-like material breaks and disappears leaving the ends free and relaxed. *C*. The same configuration of chromosomes as in *A*, after the ends have broken apart. (Sketched using an oil immersion objective.) *D*. Detailed study of the bands at the ends of the two chromosomes which were previously adherent.

One such case was found. The third involved the chromosomes separating into two groups. This was due to the fact that the bases of the arms were originally joined in two groups—one autosome being alone. Two such cases were noted. Examples of this arrangement of the chromocenter were previously observed and recorded (but not published) when uninjured nuclei in whole mount preparations were being examined for other reasons (Hinton and Atwood, 1941 and 1942). These same arrangements and variations thereof were noted frequently in the broken nuclei. While pulling adhesions apart with the microneedles, the configuration of chromosomes would often be altered, stretching out into any one of the above mentioned types. At least five examples of each type are recorded.

No claim is made that the semi-fixed chromosomes used in these experiments accurately represent the living state. The work on these chromosomes does, however, throw light on such of their physical characteristics as are involved in the preparation of aceto-orcein slides, and shows the behavior of terminal adhesions and the chromosome arrangement from the beginning of fixation in the uninjured nucleus to the time the fully fixed and smeared preparation is observed.

The data show that a terminal adhesion is a condition in which the ends of the two chromosomes are strongly held together by a non-staining material which is continuous with the ends. They also show that the connection between the ends, even though tenacious, is still the weakest place along the chromosomes, with the exception of the adhered bases of the chromosomes. But the very fact that the ends invariably held together as firmly as they did, makes it unlikely that many cases of terminal adhesions are broken apart in the preparation of a slide.

Goldschmidt and Kodani (1942, 1943) noticed "the apparent strength of the cohesion which leads to a breaking off of tips which are attached to another chromosome in chromosome smear preparations." The foregoing data show that when two adhered chromosome ends are mechanically pulled apart, the phenomenon described by Goldschmidt and Kodani does not occur. Instead, it is evident that when a chromosome is fixed to the degree that is usual in preparing a smear, the adhered ends do not break apart with any ease. Even if they do break apart, it does not result in terminal deficiencies and translocations. In addition, if this type of aberration were produced by mechanical means, for every translocation there should be a deficient tip in the same nucleus, but Goldschmidt and Kodani fail to produce such evidence. It is possible that in a more thoroughly fixed chromosome, the phenomenon as postulated by Goldschmidt and Kodani could occur, but as shown elsewhere in the present paper, the frequency of terminal adhesions in a given stock at a given temperature does not vary, regardless of the length of time of fixation. If terminal adhesions break apart in smearing more readily after extreme fixation, then the frequency of terminal adhesions found on smear preparations should vary with the degree of fixation. It seems most unlikely from the data herein described that the small terminal deficiencies and translocations as seen by Goldschmidt and Kodani could be "pseudodeficiencies and translocations produced by mechanical breaks near the chromosome tip following cohesion of the telomere at the tips of two chromosomes." It seems much more likely that the deficiencies and translocations as seen by these authors were true deficiencies and translocations carried in the stocks which they examined; and that due to the small size of these rearrangements they failed to observe them in all nuclei, for it

would be extremely difficult to detect consistently an aberration as minute as one involving only one to four bands.

The idea that these small aberrations were carried as characteristics of their stocks does not seem unlikely in light of other work on the subject. Thus Kikkawa (1938) found a number of terminal structural differences between the chromosome ends of various *Drosophila ananassae*. His drawings of these extra bands on the chromosome ends are entirely similar to the drawings presented by Goldschmidt and Kodani (1943) for *Drosophila melanogaster*. Again, Dobzhansky and Dreyfus (1943) found, in Brazilian populations of *Drosophila ananassae*, "variations apparently identical with those described by Kikkawa." Also Bridges made a study of the free ends of the salivary chromosomes of *D. melanogaster* and found various examples of this type of aberration as characteristic of different strains. Unfortunately these data were never published in full but cases are listed in *Drosophila Information Service* No. 9—Df(2)Ore-R, Df(2)Sw-L, Df(2)Sw-R, Df(3)D<sup>3</sup>H<sup>R</sup>, Df(3)Mz<sup>L</sup>. (See also Bridges and Brehme, 1944, page 59.) There seems, therefore, little doubt that the terminal aberrations reported by Goldschmidt and Kodani, Kikkawa, Dobzhansky and Dreyfus, Bridges, and others, are all of the same nature and are inherent in the chromosomes of the individual stock being studied.

That these terminal deficiencies and translocations derive from the separation of terminal adhesions between non-homologous chromosomes, not in salivary gland nuclei, but during meiosis, remains a possibility. It is not inconceivable that a salivary gland nucleus is comparable to a pachytene nucleus of the first meiotic division. If a terminal adhesion occurred during the meiotic prophase and the ends later separated unequally, then the gamete receiving one of the involved chromatids would carry to the offspring a terminal deficiency or translocation. Such meiotic behavior represents one way in which terminal adhesions could play a role in the production of terminal aberrations. Terminal adhesions of non-homologous chromosome ends during the prophase of meiosis have been observed by Slack (1938) in *Corixa*, Schrader (1941) in *Edessa irrorata*, Ribbands (1941) in *Habropogon*, Rhoades and McClintock (verbal communication) in *Zea Mays*, and others (see Ribbands, 1941). Also terminal adhesions at meiosis may conceivably be involved with the deposition of matrix substance and the polarization of the chromosomes (see Schrader, 1941).

#### THE GENETICAL NATURE OF ADHESIONS AND FACTORS INFLUENCING ADHESIONS

##### *Change of frequencies with time*

The frequency with which any two chromosome ends were associated was established for the Oregon-R strain in July 1940 (Hinton and Sparrow, 1941), and for the Swedish-b strain in July 1941 (Hinton and Atwood). These percentages are brought together in Table I for the sake of comparison. During the present investigation the frequencies in both Oregon-R and Swedish-b were reexamined (November 1943) by recording the types of adhesions observed from aceto-orcein smear preparations of glands. A marked difference is seen to exist when the current data are compared with those collected in 1940 and 1941. This difference is demonstrated in Table I. The data are from females only and are in per cents of the total cases examined in each case.

TABLE I

| Chromosome combinations | 1             | 2                | 3                 | 4             | 5                 | 6                 |
|-------------------------|---------------|------------------|-------------------|---------------|-------------------|-------------------|
|                         | Ore'R<br>7/40 | Ore'R<br>11/7/43 | Ore'R<br>11/17/43 | Sw.-b<br>7/41 | Sw.-b<br>11/28/43 | Sw.-b<br>11/30/43 |
| X-2L                    | 17.3          | 21.0             | 20.0              | 14.0          | 3.0               | 1.0               |
| X-2R                    | 3.1           | 12.0             | 4.0               | 0.9           | 10.0              | 14.0              |
| X-3L                    | 17.3          | 5.0              | 8.0               | 16.8          | 8.0               | 0.0               |
| X-3R                    | 16.3          | 20.0             | 24.0              | 5.6           | 9.0               | 21.0              |
| 2L-2R                   | 3.1           | 2.0              | 4.0               | 9.3           | 1.0               | 2.0               |
| 2L-3L                   | 3.1           | 1.0              | 2.0               | 24.3          | 3.0               | 3.0               |
| 2L-3R                   | 5.1           | 1.0              | 1.0               | 10.0          | 1.0               | 1.0               |
| 2R-3L                   | 6.1           | 1.0              | 1.0               | 8.4           | 5.0               | 3.0               |
| 2R-3R                   | 16.3          | 31.0             | 30.0              | 2.8           | 52.0              | 50.0              |
| 3L-3R                   | 12.2          | 6.0              | 6.0               | 7.5           | 8.0               | 5.0               |
| Total cases             | 98            | 100              | 100               | 107           | 100               | 100               |

It can be concluded from the data in Table I that:

1. The frequencies within each set of data are non-random. (Chi-square tests give probabilities of much less than .01 in each case that the data are similar to the expected random distributions.)
2. Oregon-R and Swedish-b in 1940-41 differed significantly from each other. (P—less than .01.)
3. Oregon-R, 1943, differs significantly from Swedish-b 1943. (P—less than .01.)
4. Oregon-R, 1940, differs significantly from Oregon-R 1943 (columns 2 and 3). (P—.01 to .02.)
5. Oregon-R, 11/9/43, does not differ significantly from Oregon-R, 11/17/43. (P—.70.)
6. Swedish-b, 1941, differs significantly from Swedish-b, 1943 (columns 5 and 6). (P—less than .01.)
7. Swedish-b, 11/28/43, does not differ significantly from Swedish-b, 11/30/43. (P—.05 to .10.)

The fact that both the Oregon-R and the Swedish-b patterns of adhesions have changed over a three year period of time suggests some mechanism for rapid evolution of the factors involved in terminal adhesions. The possibility exists that the stocks in question are not the identical stocks used in 1940-1941. However, since both Oregon-R and Swedish-b have changed, this seems less likely than if only one stock had changed.

### *Dominance*

It was shown (Hinton and Atwood, 1941 and 1942) that when Swedish-b females were crossed to Oregon-R males, the  $F_1$  females were identical with the Oregon-R parents in the relative frequencies of types of adhesions. This was interpreted to mean that the factors controlling the Oregon-R pattern of adhesions were in some way entirely dominant to the Swedish-b. Since, in the present investigation it has been shown that both the Oregon-R and Swedish-b pattern have

changed over a three year period, it was deemed advisable to reexamine the heterozygote (Ore'R/Sw.b). The results are presented in Table II.

The probability that the heterozygote has an adhesion pattern resembling that of the Swedish-b chromosomes is .10 as shown by an  $\chi^2$  test. However, the probability that the heterozygote shows the same pattern as the Oregon-R parent is much greater ( $P=.50$ ); yet, at the same time, an equal probability (.50) is shown that the heterozygote resembles the intermediate between the two parental patterns. Therefore it seems impossible at this time to conclude whether dominance has been maintained by Oregon-R or whether the dominance has been lost and Oregon-R and Swedish-b now have an equal ability to determine the adhesion pattern of the  $F_1$ , and either modify or compensate each other. However, if an examination is made of the only two classes wherein Oregon-R and Swedish-b are really dissimilar (X-2L and 2R-3R), then an  $\chi^2$  test shows the following: the proba-

TABLE II  
*Females, 1943*

| Chromosome combinations | Per cent adhesions Ore'R | Per cent adhesions Ore'R/Sw.-b | Per cent adhesions Sw.-b |
|-------------------------|--------------------------|--------------------------------|--------------------------|
| X-2L                    | 20.5                     | 12.5                           | 2.0                      |
| X-2R                    | 8.0                      | 15.5                           | 12.0                     |
| X-3L                    | 6.5                      | 6.5                            | 4.0                      |
| X-3R                    | 22.0                     | 16.0                           | 15.0                     |
| 2L-2R                   | 3.0                      | 3.0                            | 1.5                      |
| 2L-3L                   | 1.5                      | 2.0                            | 3.0                      |
| 2L-3R                   | 1.0                      | 2.0                            | 1.0                      |
| 2R-3L                   | 1.0                      | 1.0                            | 4.0                      |
| 2R-3R                   | 30.5                     | 39.0                           | 51.0                     |
| 3L-3R                   | 6.0                      | 2.5                            | 6.5                      |
| Total cases             | 200                      | 200                            | 200                      |

bility that Ore'R and Ore'R/Sw.-b are similar—.10; that Sw.-b and Ore'R/Sw.-b are similar—less than .01; and that Ore'R/Sw.-b and the theoretical intermediate between Ore'R and Sw.-b are similar—.70. It would appear from this analysis that Oregon-R has lost its dominance completely and that the heterozygote is merely the intermediate between the two parental strains in its adhesion pattern.

#### *Cytoplasmic effect*

In order to observe the pattern of adhesions of identical chromosomes in different cytoplasm, the reciprocal crosses between Oregon-R and Swedish-b were made. Swedish-b females crossed to Oregon-R males produce  $F_1$  daughters heterozygous for a complete set of Oregon-R chromosomes and a set of Swedish-b chromosomes, in Swedish-b cytoplasm (Ore'R/Sw.-b). Swedish-b males crossed to Oregon-R females give identical results with the exception that the cytoplasm is Oregon-R. The data from these two types of heterozygous daughters are presented in Table III.

The data show that the cytoplasm probably has no effect on the frequency of types of adhesions. An  $\chi^2$  test gives a probability of .10 that the two sets of

data are similar. But here again, if the two crucial classes (X-2L and 2R-3R) are examined, they are exactly similar in the two sets of data. Therefore the factors which may be responsible for the specificities shown in terminal adhesions appear to be characteristic of the chromosomes and not dependent upon the genotype of the cytoplasm. This is in agreement with the conclusion reached by Hinton and Atwood (1941).

### *Temperature effect*

A preliminary experiment indicated that temperature has an effect on the total incidence of terminal adhesions. By counting the number of nuclei examined and the number of terminal adhesions found, it was discovered that if the culture bottles remained at 17° during the development of the larvae approximately 20 per cent of the nuclei contained a terminal adhesion; if the culture bottle remained at

TABLE III

| Chromosome combinations | Per cent adhesions Ore' R/Sw.-b in Ore' R cytoplasm | Per cent adhesions Ore' R/Sw.-b in Sw.-b cytoplasm |
|-------------------------|---|--|
| X-2L                    | 13  | 12   |
| X-2R                    | 18  | 13   |
| X-3L                    | 5   | 8  |
| X-3R                    | 19  | 13   |
| 2L-2R                   | 1   | 5  |
| 2L-3L                   | 0   | 4  |
| 2L-3R                   | 3   | 1  |
| 2R-3L                   | 1   | 1  |
| 2R-3R                   | 39  | 39   |
| 3L-3R                   | 1   | 4  |
| Total cases             | 100   | 100  |

23°, only 13 per cent of the nuclei contained an adhesion. In order to verify this conclusion the experiment was repeated with an attempt to collect more exact data. Females were allowed to lay eggs for 24 hours in culture bottles placed at 17°, and the flies then discarded. A second set of culture bottles received the same treatment at 23°. The culture bottles remained at their respective temperatures until the larvae were ready to pupate, at which time salivary gland preparations were obtained. Fifty figures were examined and the per cent of figures containing adhesions was calculated. Five such sets of data were collected from the cultures at 17°, and three from the cultures at 23°. The average percentage of adhesions per 50 nuclei occurring at 17° differs significantly from that at 23°. At 17° the mean percentage was 20 with a variation of  $\pm 6$  per cent (14-26 per cent); at 23°,  $13 \pm 4$  (9-17 per cent). The conclusion seems justified that the frequency of terminal adhesions varies with temperature.

To determine during which period of larval development temperature has its effect, culture bottles were placed at 23° for 24, 48, 72, and 96 hours respectively, and then removed to 17° where they remained until the larvae were ready to pupate. It can be seen from Figure 2 that there is little effect from the tempera-

ture as long as the larvae spend no more than the first three days of their development at 23°. After that the percentage of adhesions rapidly decreases. There are two ways to interpret this decrease in the frequency of adhesions: either terminal adhesions are sensitive to temperature during the fourth day of larval development; or there is an accumulative effect of temperature requiring at least three days. Further experimentation will make it possible to select between these two possibilities, but at this time only these preliminary data on temperature effect have been completed.

It was suspected that some cytological phenomenon could be correlated with the temperature effect. Salivary gland preparations were obtained from second instar larvae (the stage reached at the end of the third day at 23°). This preliminary cytological investigation failed to reveal a clear picture of the chromosomes since

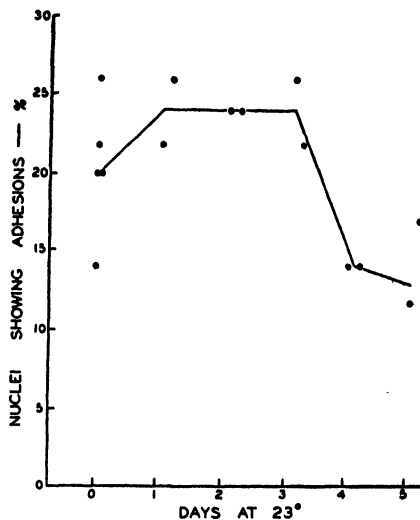


FIGURE 2.

they are most difficult to interpret at this early stage. It will be necessary to employ various cytological techniques before the question can be entirely settled. The results of the preliminary investigation do not exclude the possibility that terminal adhesions have not formed at the time of the second instar.

It will be noted, Figure 2, that the frequency of adhesions is slightly greater when the larvae are placed at 23° for the first several days and then returned to 17° than when the larvae undergo their entire development at 17°. If this is a significant difference, and only more detailed analysis will clarify that, then an explanation becomes difficult. It would indicate that there may be two occasions during the development of the chromosomes when temperature can effect terminal adhesion formation, or that there may be two different ways by which temperature exerts its effect.

In order to locate 100 terminal adhesions in the Swedish-b strain in cultures raised at 17° it was necessary to study, on the average, 45 smear preparations of pairs of salivary glands; but in order to locate 100 adhesions in the same strain

raised at 23°, it was necessary to study 128 smear preparations. Yet, when the 100 adhesions from 45 smear preparations are compared as to types of adhesions with the 100 taken from 128 preparations, it is apparent that the terminal adhesion pattern (specificities) remains unaltered. This is demonstrated by the data in Table IV. An  $\chi^2$  test gives the following probabilities that these sets of data are similar: Column A compared to column C —  $P = .80$ ; A to D = .80; A. to E = .95; B to C = .60; B to D = .05; B to E = .02; columns A plus B compared to C —  $P = .99$ ; AB to D = .70; AB to E = .50; and columns A plus B compared to columns C plus D plus E —  $P = .70$ . Therefore, it can be concluded that temperature does not affect the types of adhesions which occur, but only the total incidence of adhesions.

TABLE IV

| Chromosome combinations | Per cent adhesions<br>Sw.-b 17° |     | Per cent adhesions<br>Sw.-b 23° |     |     |
|-------------------------|---------------------------------|-----|---------------------------------|-----|-----|
|                         | A                               | B   | C                               | D   | E   |
| X-2L                    | 3                               | 1   | 3                               | 3   | 2   |
| X-2R                    | 10                              | 14  | 14                              | 7   | 8   |
| X-3L                    | 8                               | 0   | 5                               | 8   | 10  |
| X-3R                    | 9                               | 21  | 17                              | 13  | 8   |
| 2L-2R                   | 1                               | 2   | 2                               | 3   | 3   |
| 2L-3L                   | 3                               | 3   | 2                               | 2   | 2   |
| 2L-3R                   | 1                               | 1   | 1                               | 4   | 3   |
| 2R-3L                   | 5                               | 3   | 5                               | 5   | 7   |
| 2R-3R                   | 52                              | 50  | 46                              | 46  | 48  |
| 3L-3R                   | 8                               | 5   | 5                               | 9   | 9   |
| Total cases             | 100                             | 100 | 100                             | 100 | 100 |

### *Intercalary adhesions*

The ends of the chromosomes not only adhere to each other, but are also found adhered to regions along the length of the chromosome arms or to the bases of arms. Preliminary data on intercalary adhesions were reported by Hinton and Atwood (1941). Since that time 180 cases have been recorded which represent sufficient data to justify an analysis. All of these data were used to plot the frequency with which the various chromosome regions were involved in adhesions and this was compared with values expected on a random basis (number of adhesions divided by the number of chromosome regions as shown on Bridges map—100). A striking deviation from randomness was apparent, with some regions containing as high as 12 per cent of the total cases found, while in other places along the chromosomes as many as five consecutive regions contained no adhesions. In order to treat the data statistically in the most simple manner, each of the five long chromosome arms was divided into four parts by using every five regions on the Bridges map as a unit. These 20 sections were then compared with the theoretical random distribution. An  $\chi^2$  test gave a probability of less than .01 that the adhesions could be distributed along the chromosomes at random. The conclusion is justified that there are certain regions of the chromosomes which are

associated in adhesions with a high frequency, while the majority of regions show the expected frequency.

The more distal regions of the chromosome arms show a higher frequency of adhesions than do the more proximal. This is best illustrated by Figure 3 in which are shown the five chromosome arms divided into four regions and plotted against the percentage of adhesions occurring per region. The data pictured in Figure 3 involve only intercalary regions, the data for the tips adhered to these regions being reserved for separate analysis.

Since the distal fourth of each chromosome arm shows a greater frequency of adhesions, it is necessary to assume, either that any material responsible for this type of pairing is present in greater abundance near the ends of the chromosome, or that due to the arrangement of the chromosomes, the distal regions are more accessible to the tips of other chromosomes. If the first hypothesis were true and it be postulated that this type of pairing is due to the attraction of heterochromatin, than a greater amount of intercalary heterochromatin should be located in the distal quarter of each chromosome arm than in any other region. There is no cytological evidence to support such an assumption, but since some heterochromatin regions present in dividing chromosomes do not appear in salivary gland chromosomes (Hinton, 1942) such a possibility is not eliminated. If the second hypothesis were true then the differential frequency would be due solely to mechanical causes.

Those regions which have a strikingly high occurrence of adhesions are shown in the following list:

| Chromosome arm | Regions        |
|----------------|----------------|
| X              | 2B, 3C         |
| 2L             | 21/22          |
| 2R             | 56, 58         |
| 3L             | —              |
| 3R             | 99/100, 100C/D |

Sixty-six additional places along the length of the chromosome were found to be involved in adhesions, but none of these with a surprisingly high frequency. Kaufmann (1939b) studied the position of X-ray induced breaks along the X chromosome and discovered at least 12 regions with a breakage frequency considerably higher than the expected frequency of a random distribution. He suggested that these regions contain heterochromatin which would account for the high breakage by X-rays and for the fact that these regions occasionally pair with each other and with the chromocenter. The question arises: Are the regions which show a high frequency of adhesions correlated with those showing a high breakage frequency? Table V, shown on the next page, makes such a comparison.

It is true that some regions with a high breakage frequency are found to be involved in adhesions, but on the whole neither a positive nor a negative correlation exists. Since those regions which have the highest adhesion frequency are not the same regions which show a high breakage frequency, non-specific heterochromatin certainly cannot be the important factor in both cases, although the possibility is not eliminated that it is important in one case or the other. Such a comparison as this is justified since the data indicate that neither the breakage frequency (Kaufmann, 1939b) nor the adhesion frequency (see following paragraph) varies for a given region in various strains.

Since it had been demonstrated that the pattern of end-to-end adhesions is different in different strains, the question arises as to whether the adhesion involving intercalary regions also express different specificities in different strains. It is difficult to answer this question definitely due to the relative paucity of data as compared to the several thousand places along the chromosomes that adhesions might occur, but in the Oregon-R strain 69 cases of intercalary adhesions have been recorded; and 46 in the Swedish-b strain, and when these cases are plotted against

TABLE V

| Regions found<br>by Kaufmann<br>to have a high<br>breakage<br>frequency | Regions found by<br>Hinton to be involved<br>in adhesions |                        |
|---|---|------------------------|
|   | Region  | Number of<br>adhesions |
| 1F  | 1C/D  | 1                      |
|   | 2B  | 11                     |
|   | 2D  | 1                      |
|   | 3B  | 1                      |
|   | 3C  | 22                     |
|   | 3D  | 1                      |
|   | 4B  | 2                      |
| 4E  | 5   | 3                      |
|   | 5/6   | 2                      |
|   | 7B  | 1                      |
| 7B  |   |                        |
| 7C  |   |                        |
| 8B  |   |                        |
|   | 8F  | 1                      |
|   | 8/9   | 1                      |
|   | 9   | 2                      |
|   | 10A   | 1                      |
| 11A   | 11A   | 1                      |
| 12D   | 12D   | 2                      |
| 12E   |   |                        |
|   | 14  | 1                      |
|   | 15/16   | 1                      |
| 16E   | 16E   | 1                      |
| 16F   |   |                        |
|   | 18E/F   | 1                      |
| 19E   | 19E   | 2                      |
| 19F   |   |                        |
|   | 20B   | 1                      |

chromosome regions and the two sets compared it can be observed that the regions showing high frequency of adhesions are usually the same in each case and that the distribution is in general very similar. At least from the data on hand the indications are that even though intercalary adhesions are not at random, the specificity remains constant from strain to strain.

So far this discussion of intercalary adhesions has dealt only with the sub-terminal regions involved in the adhesion with no regard to which chromosome ends were involved. Data are presented in Table VI showing the number of times

each chromosome end is involved in an intercalary adhesion (in per cent of the number of cases observed).

From these data a difference in the behavior of the ends in the two strains is apparent. An  $\chi^2$  test gives a probability of less than .01 that the two sets of data are similar. It therefore appears that the intercalary regions involved in adhesions remain the same from one strain to another but the chromosome ends involved vary from one strain to another. Another point of interest in these data is the correlation between the behavior of chromosome ends involved in intercalary ad-

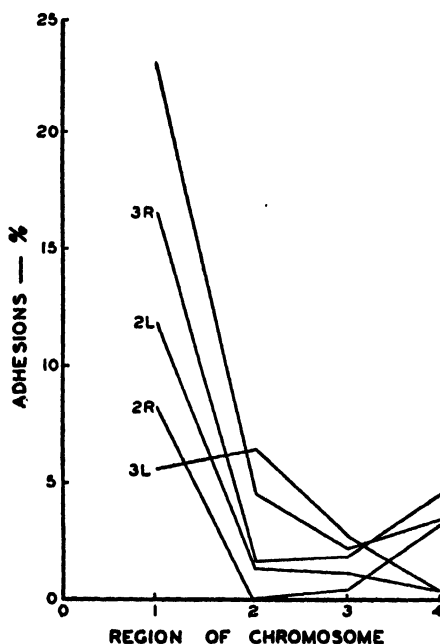


FIGURE 3. The number of intercalary adhesions is plotted against chromosome regions. Region 1 is the most distal in each case, but does not include the tip of the chromosome. Data for tips involved in intercalary adhesions are presented in Table VI.

hesions and those involved in terminal adhesions. It may be recalled that in Swedish-b, 2R and 3R adhere together with a frequency much greater than they do in Oregon-R. In Table VI it can be seen that both 2R and 3R are involved in intercalary adhesions with a frequency higher in Swedish-b than in Oregon-R. The same correlation exists for 2L and 3R. The only chromosome end which behaves the same in terminal adhesions in both strains is 3L, and it is found also to be involved in intercalary adhesions with about equal frequency in both strains (8 per cent and 6 per cent). A correlation is indicated between the potentialities of a chromosome end participating in terminal adhesions and in intercalary adhesions.

#### *Chromosome substitution*

The distinguishing feature of the adhesion pattern in the Oregon-R strain as contrasted with the Swedish-b lies in the considerably higher frequency of the X-2L

combination of chromosome ends (20.5 per cent as compared to 2.0 per cent) and lesser frequency of the 2R-3R combination (30.5 per cent as compared to 51.0 per cent). The eight other possible combinations of two chromosome ends occur with an approximately equal frequency in both strains. What will be the reaction of the Swedish-b X (which adheres to Sw.-b 2L in only 2 per cent of the adhesions observed) with an Oregon-R 2L that is accustomed to adhering to X in 20.5 per cent of the cases? The following experiment was designed to test the effect of a Sw.-b X chromosome upon the adhesion pattern of the Ore'R chromosome by substituting a Sw.-b X for an Ore'R X, leaving the autosomes entirely Ore'R. In order to achieve the substitution of one chromosome in a strain it is necessary genetically to mark each chromosome in such a manner that it can be identified and also to prohibit crossing over so that no alteration in the chromosomes can

TABLE VI

| Chromosome end | End involved in intercalary |       | End involved with end |       |
|----------------|-----------------------------|-------|-----------------------|-------|
|                | Strain                      |       | Strain                |       |
|                | Ore'R                       | Sw.-b | Ore'R                 | Sw.-b |
| X              | 54                          | 35    | 28                    | 16    |
| 2L             | 7                           | 3     | 13                    | 4     |
| 2R             | 26                          | 41    | 21                    | 34    |
| 3L             | 8                           | 6     | 7                     | 8     |
| 3R             | 4                           | 15    | 29                    | 38    |

occur. If this is achieved, the result should be a strain of *Drosophila* entirely homozygous for Ore'R chromosomes with the exception of the X chromosomes which are homozygous Sw.-b. The obvious objection was foreseen, that regardless of the results of such a substitution no distinction could be made between the effect of the foreign chromosome as a whole and the effect of merely the foreign tip. It was therefore necessary to compare simultaneously a stock homozygous for Ore'R chromosomes with the exception of the tip of the X chromosome which would be homozygous Sw.-b. The creation of such a stock required crossing over at the tip of the X chromosome but under such conditions that the entire complement of chromosomes would remain Ore'R with the exception of this small terminal region. As a control to compare with these two altered stocks, it was necessary to recover from the crosses a stock carrying a complete homozygous set of Ore'R chromosomes. These three stocks which the experiment is designed to create may be pictured diagrammatically as follows (the small fourth chromosome is ignored in these experiments):

|    | X chromosome |           | Chromosome 2 and 3 |       |
|----|--------------|-----------|--------------------|-------|
|    | Tip          | Remainder |                    |       |
| 1. | Ore'R        | Ore'R     | Ore'R              | Ore'R |
| 2. | Sw.-b        | Ore'R     | Ore'R              | Ore'R |
| 3. | Sw.-b        | Sw.-b     | Ore'R              | Ore'R |

The genetical technique used to achieve these three stocks follows: In order to maintain the autosomes from one generation to another without losing their iden-

tity, chromosomes with dominant markers and inversions in both arms were carried heterozygously with the wild-type chromosomes (Cy al<sup>2</sup> L<sup>4</sup> sp<sup>2</sup> and Mé, In (3R)C, Sb e<sub>1</sub> (3) e). For ease in referring to these stocks, they are abbreviated in this paper as Cy L and Mé Sb, respectively. In order to obtain the proper crossover near the tip, an X chromosome marked with recessive mutants was used (sc ec cv ct<sup>6</sup> v s<sup>2</sup> f car bb<sup>1</sup> (abbreviated sc ec cv . . .)). A crossover between scute (sc) and echinus (ec) would be in the 2 or 3 region of the salivary map or distal to 5.5 on the genetical map. This is relatively near the end of the X and would leave about 9/10th of the chromosome intact with only the distal 1/10th replaced. Other stocks used as markers were: y Hw d149 m<sup>2</sup> g<sup>4</sup> (abbreviated d149), Cl B, and Cy/Pm, ds<sup>39k</sup>; H/C, Sb (abbreviated Cy/Pm; H/Sb). The series of crosses is summarized in Table VII.

TABLE VII

|                |   |  |   |   |                                     |
|----------------|---|--|---|---|-------------------------------------|
| P <sub>1</sub> | Sw.-b   | x  | sc ec cv . . .  | x   | Ore'R                               |
| F <sub>1</sub> | $\frac{sc\ ec\ cv\ \dots}{Sw.-b}$   | x  | sc ec cv . . .  | $\frac{sc\ ec\ cv\ \dots}{Ore'R}$   | x $\frac{Cy}{Pm}; \frac{H}{Sb}$     |
| F <sub>2</sub> | $\frac{Sw.-b - ec\ cv\ \dots}{sc\ ec\ cv\ \dots}$                               | x  | sc - Ore'R; Pm; H   |   |                                     |
| F <sub>3</sub> | sc ec cv . . .  | x  | $\frac{Sw.-b - ec\ cv\ \dots}{sc - Ore'R}; Pm; H$                       | x   | Ore'R                               |
| F <sub>4</sub> | Ore'R   | x $\frac{Sw.-b - Ore'R}{sc\ ec\ cv\ \dots}; Pm; H$ | d149; Cy L; Mé Sb   | x $\frac{Sw.-b - Ore'R}{d149}; \frac{Cy\ L}{Ore'R}; \frac{Mé\ Sb}{Ore'R}$ | $\frac{Pm}{Ore'R}; \frac{H}{Ore'R}$ |
| F <sub>5</sub> | $\frac{Sw.-b - Ore'R}{Ore'R}; \frac{Pm}{Ore'R}; \frac{H}{Ore'R}$                | x  | $\frac{Sw.-b - Ore'R}{d149}; \frac{Cy\ L}{Ore'R}; \frac{Mé\ Sb}{Ore'R}$ |   |                                     |
| F <sub>6</sub> | $\frac{Sw.-b - Ore'R}{Sw.-b - Ore'R}; \frac{Ore'R}{Ore'R}; \frac{Ore'R}{Ore'R}$ |  |   |   |                                     |

In order to establish a stock which has an entire Sw.-b X chromosome with Ore'R autosomes, and a control stock with a full complement of Ore'R chromosomes, the procedure diagrammed in Table VIII was used.

TABLE VIII

|                |   |   |   |   |   |  |
|----------------|---|---|---|---|---|--|
| P <sub>1</sub> | Sw.-b   | x | d149; Cy L; Mé Sb   | d149; Pm; H   | x | Ore'R  |
| F <sub>1</sub> | $\frac{d149}{Sw.-b}; \frac{Cy\ L}{Sw.-b}; \frac{Mé\ Sb}{Sw.-b}$ | x | Ore'R   | $\frac{d149}{Sw.-b}; \frac{Cy\ L}{Sw.-b}; \frac{Mé\ Sb}{Sw.-b}$ | x | $\frac{Pm}{Ore'R}; \frac{H}{Ore'R}$                              |
| F <sub>2</sub> | $\frac{d149}{Ore'R}; \frac{Cy\ L}{Ore'R}; \frac{Mé\ Sb}{Ore'R}$ | x | $\frac{Ore'R}{Ore'R}; \frac{Ore'R}{Ore'R}; \frac{Ore'R}{Ore'R}$ | $\frac{d149}{Sw.-b}; \frac{Cy\ L}{Ore'R}; \frac{Mé\ Sb}{Ore'R}$ | x | $\frac{Sw.-b}{Ore'R}; \frac{Cy\ L}{Ore'R}; \frac{Mé\ Sb}{Ore'R}$ |
| F <sub>3</sub> | $\frac{Ore'R}{Ore'R}; \frac{Ore'R}{Ore'R}; \frac{Ore'R}{Ore'R}$ |   |   | $\frac{Sw.-b}{Sw.-b}; \frac{Ore'R}{Ore'R}; \frac{Ore'R}{Ore'R}$ |   |  |

After these three stocks were obtained, salivary smear preparations were made and 100 cases of terminal adhesions recorded from each. Table IX presents the data comparing the effect on the Ore'R pattern of terminal adhesions of a Sw.-b X chromosome versus a Sw.-b X chromosome tip versus an Ore'R X chromosome.

An examination of the data shows that the Sw.-b X (which seldom adheres with a Sw.-b 2L) when placed with an Ore'R 2L (which normally adheres frequently with Ore'R X) seldom adheres with it (column 2, Table IX). This introduced chromosome brings with it into the Ore'R strain the specificities which characterized it in the Sw.-b strain. Therefore the tendency of a chromosome to adhere or not to another chromosome is a property of the chromosome itself and not of the group of chromosomes as a whole.

But the experiment also makes possible a deeper analysis of chromosome behavior. When we are dealing with a chromosome complement of Ore'R in which only the tip of the X chromosome has been derived from the Sw.-b strain, there is still little adhesion between such an X and the left arm of chromosome 2 (column 3, Table IX). The strong attraction between the X-chromosome and 2L which is so typical of the Ore'R stock thus is abrogated by the substitution of a very small terminal portion of the X from the Sw.-b strain. It is therefore in the tip region

TABLE IX

| Chromosome combinations | 1<br>Ore'R X with<br>Ore'R autosomes | 2<br>Sw.-b X with<br>Ore'R autosomes | 3<br>Sw.-b tip on<br>Ore'R X with<br>Ore'R autosomes | 4<br>Sw.-b X with<br>Sw.-b autosomes |
|-------------------------|--------------------------------------|--------------------------------------|--|--------------------------------------|
| X-2L                    | 17                                   | 1                                    | 1  | 1                                    |
| X-2R                    | 4                                    | 1                                    | 6  | 14                                   |
| X-3L                    | 10                                   | 7                                    | 5  | 0                                    |
| X-3R                    | 20                                   | 9                                    | 0  | 21                                   |
| 2L-2R                   | 2                                    | 5                                    | 3  | 2                                    |
| 2L-3L                   | 3                                    | 8                                    | 10   | 3                                    |
| 2L-3R                   | 3                                    | 2                                    | 5  | 1                                    |
| 2R-3L                   | 5                                    | 7                                    | 8  | 3                                    |
| 2R-3R                   | 30                                   | 36                                   | 39   | 50                                   |
| 3L-3R                   | 6                                    | 24                                   | 23   | 5                                    |
| Total cases             | 100                                  | 100                                  | 100  | 100                                  |

of the chromosome that its adhesion properties are localized and the body of the chromosome as a whole is not responsible for the effect.

Finally it is to be noted that the X from Sw.-b (be it the whole X or only the tip replacing the Ore'R tip) fails to adhere frequently also to 3R of Ore'R. Since the X chromosomes of both stocks have a strong tendency to adhere to 3R if the latter is from the same stock, this must mean that the properties involved in the X-3R combination in one stock are different from those involved in the other stock.

Since two of the most frequent types of adhesions (X-2L; X-3R) are practically eliminated by introducing a Sw.-b X (or tip) into the Ore'R environment, it would be expected that the total frequency of adhesions should be reduced. This is not the case. The total number of adhesions per nucleus remains the same as in the parental strains (22 per cent of the nuclei contain adhesions in the Sw.-b tip stock; 20 per cent in the parental stocks). Thus the other eight possible combinations of chromosome ends (two-by-two) must rise in frequency to compensate for the two missing categories. Do they rise proportionally as might be expected? The answer is clearly that they do not rise proportionately. While some combinations

occur with an unaltered frequency, others are encountered much more often, and notably 3L-3R which soars from 6 per cent to 24 per cent. The indications seem to be that 3L and 3R, since they remain structurally unaltered, had the ability to adhere in the Ore'R strain but this was never realized due to a stronger attraction between X and 3R. In other words, once the stronger attraction is eliminated by bringing in a foreign X, the weaker attraction becomes the strongest remaining. (The word "attraction" is used throughout in a purely descriptive sense and in no way is an attempt made to imply physical forces.)

Thus, by substituting a foreign X chromosome and a foreign X chromosome tip into the Ore' R strain, the locus of specificity involved in terminal adhesion is found to be the end of the chromosome. The experiment also reveals unsuspected

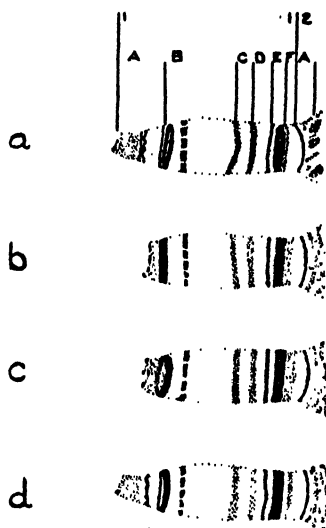


FIGURE 4. The distal end of the X chromosome in: a. Oregon-R. b. Swedish-b. c. Swedish-b on Oregon-R base. d. Marker stock.

potential attractions carried by the chromosome ends—attractions which result in adhesions only in the absence of stronger attractions.

#### *Cytological examination of the chromosome ends*

Detailed study was made of the banding at the end of the chromosome in the Sw.-b strain, the Ore'R strain, the Sw.-b tip on the Ore'R X base, and the marker stock used to obtain the crossovers (*sc ec cv ct<sup>6</sup> v s<sup>2</sup> f car bb<sup>1</sup>*). This study demonstrated that the Ore'R and the marker stock X chromosomes are longer by a noticeable amount than either of the two Sw.-b X chromosomes. A study of Figure 4 shows that the Ore'R and marker X chromosomes have regions 1A 1-2-3 (Bridges map) while the others lack this. Whether this is a typical terminal deficiency as described in connection with the micromanipulation data in a previous section of the present paper, or not, the fact remains that there is a definite morphological difference in the X chromosome tips in these strains and furnishes

evidence in addition to the genetical evidence that the tip of the experimental stock is truly Sw.-b. It becomes obvious to suggest that the different specificities in terminal adhesion shown by the Ore'R and Sw.-b chromosomes may be due to the morphological difference. It was previously suggested (Hinton and Atwood, 1941) after a study of adhesions in a terminally deficient stock, that there are sub-terminal layers of properties which can operate if the tip is removed and the raw end heals. The new end has the ability to assume the role of the previous end, but not with the identical specificities.

### DISCUSSION

Since all cells of an individual have the same descent one need not look to a difference between the chromosomes in order to explain the differences in adhesions among the cells of a single gland. Yet we also know from the experiments in the present paper that the end of the chromosome is the important factor in deciding the types of adhesions. This has meaning if we conceive of the tip as containing several properties for sticking, one of which, for example, involves a strong attraction for some other chromosome while another properly involves a weak attraction for still a different chromosome. If, at some stage in the development of the salivary gland, there is a movement of the chromosomes and an approximately equal chance that any chromosome end might pass close to any other chromosome end in the nucleus, it is logical that the two ends which have a stronger attraction for each other will show adhesion more often than will two ends with a weak mutual attraction which necessitates a closer approach before sticking can occur. This is suggesting that the different properties at the end of the chromosome responsible for the sticking have different sensitive volumes, or spheres of influence in which they are capable of attracting.

The tip replacement experiment furnishes the best clues as to the number of properties which must be assumed to be at the end of the chromosome. It is obvious from the data that all tips do not contain the same properties even though any two have some in common. Also, one end may exert an influence over another end so strong that weaker attractions are seldom realized except in the absence of the stronger attraction. Therefore the chromosome end must be assumed to contain properties seldom expressed due to the presence of more dominant properties. With these indications in mind it is possible to construct a model of the ends and the properties they contain. If the ends in Ore'R are designated as X-CA, 2L-CD, 2R-Bad, 3L-ac, 3-R-ABc; and if it be assumed that the letters used represent properties of the chromosome ends, and that the attraction between A and A is greater than between A and a which in turn is greater than between a and a, etc.; then the model expresses the various frequencies shown by the data.

Such a model is mainly a diagrammatic restatement of the data, but its main value is to indicate the amount of assumption necessary for a working hypothesis. According to this most simple model it is necessary to assume three or four properties per tip in order to include all data, and at least six different properties. One of these properties may be chromosome length, and another a general attraction between all parts of the chromosomes, but the rest of the properties must be assumed to be more specific in nature. These properties can be assumed to be either

TABLE X

(Assuming CC produced 20 per cent adhesion; Cc, 1 per cent; cc, 1; AA, 20; Aa, 6; aa, 1; BB, 20; dd, 3.)

| Combination | Attraction due to: | Frequency |        |
|-------------|--------------------|-----------|--------|
|             |                    | Expected  | Actual |
| X-2L        | CC                 | 20        | 20     |
| X-2R        | Aa                 | 6         | 8      |
| X-3L        | CC, Aa             | 7         | 6      |
| X-3R        | AA, Cc             | 21        | 22     |
| 2L-2R       | dd                 | 3         | 3      |
| 2L-3L       | Cc                 | 1         | 1      |
| 2L-3R       | Cc                 | 1         | 1      |
| 2R-3L       | aa                 | 1         | 1      |
| 2R-3R       | BB, Aa             | 26        | 30     |
| 3L-3R       | Aa, cc             | 7         | 6      |

characteristics of a single substance or units of material at the end of the chromosome.

The idea that the whole phenomenon of terminal adhesions is due to a single heterochromatic attraction becomes impossible in light of the data presented in this paper. If quantitative differences in a single factor were responsible for the different frequencies of adhesions, as is assumed by the heterochromatic theory, then a mathematical model could be set up to fit. Such a model is impossible to formulate for one cannot explain in simple quantitative terms (with the adhesion frequencies following either the sum or the product of varying quantities) such frequencies of combinations of chromosome ends as have been shown to exist by the data. If heterochromatin is the substance involved in the formation of terminal adhesions, it becomes necessary to assume at least six types of heterochromatin either with stronger attractions between some types than between others or any one type possessing several properties. The same objection holds for the telomere theory. The telomere would have to consist of many parts each with different specificities. But regardless of whether we conceive of the end of the chromosome as being heterochromatins, compound telomere, or genic material, the indications are that it shows several specific attractions of various strengths, dependent upon proximity for expression, subject to change by mutation, and able to reform if removed or altered.

#### SUMMARY

In order to obtain further insight into the nature of chromosome ends, the phenomenon of terminal adhesions in salivary gland chromosomes was studied.

Salivary gland chromosomes involved in terminal adhesions were stretched with the needles of a micromanipulator until the ends broke apart. No case was found in which the ends had gained or lost a band.

From determining which chromosomes were involved most frequently in adhesions, it is concluded that the frequency with which any two chromosome ends adhere together is non-random, differs in different strains, is subject to change

over a period of time, is not affected by the genotype of the cytoplasm nor temperature. However, the total incidence of adhesion is affected by temperature.

Intercalary regions associated with chromosome ends were found to be non-random.

A foreign chromosome end introduced into a strain demonstrates that the specificities shown in terminal adhesions are characteristics of the end itself, and reveals potential abilities of ends which are not realized under normal conditions.

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# THE ARMY-ANT BEHAVIOR PATTERN: NOMAD-STATARY RELATIONS IN THE SWARMERS AND THE PROBLEM OF MIGRATION

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The problem of migration occupies a position of considerable importance in the study of animal psychology, involving as it does questions such as "instinct," reproductive functions influencing behavior, sensory and ecological factors in behavior, and others of significance. Numerous writers have suggested, with reason, that the scientific explanation of animal migration may be improved not only through further investigations of the best known migrants, among birds and fishes, but also by studying other instances wherever they arise in the phyletic series. By investigating migration and related patterns in a variety of animal types, it may well be possible to clarify both the essential fundamental characteristics of migration and characteristics which may differ or be absent according to the given biological setting. Accordingly, our present discussion introduces such an exercise in the study of one of the social insects.

The most characteristically pedestrian of all insects, species of the ant subfamily Dorylinae in the American tropics, commonly known as "army ants," seem committed to a wandering of their colonies without any possibility of more than relatively brief nesting stops. In this respect they resemble their Old World relatives, the "driver ants," of which Savage (1847) said:

"From its locomotive habits the impression . . . has obtained, that it has no fixed habitation. This my observations go to confirm in respect both to their appearing and disappearing from certain localities, and the absence of cells or magazines."

The typical inability of doryline colonies to settle down permanently has prompted many writers (in particular: Sumichrast, 1868; Belt, 1874; Vosseler, 1905; and Wheeler, 1910) to refer to these ants as "migratory." It is in order to inquire whether the application of this term to doryline colony movements is merely gratuitous, as other writers such as Heape (1931) maintain, or is actually justified in a technical sense.

Systematic observations soon reveal that the rambling of doryline colonies is not random or haphazard but instead involves a predictable routine describable as a regular pattern. In a previous paper (Schneirla, 1938) such a pattern has

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been described for *Eciton hamatum*, the type species of the American subgenus *Eciton s.str.* In the season of rains, evidently the time of optimal *Eciton* activity, this pattern exhibits two prominent activity phases: one, a nomadic period, in which there are large daily raids, each passing at nightfall into a movement of the entire colony to a new temporary nesting site, and two, a statary period, in which small daily raids are staged from a stable bivouac site, without any bivouac-change movements. These major changes in colony behavior occur in regular succession during the rainy season, under conditions indicating that the key factor in the behavior cycle of a colony must be present or absent in dependence upon the condition of developing broods. The pace-setting basis of the entire system of events may be traced to the reproductive properties of the *E. hamatum* queen (Schneirla, 1944b).

To what extent does this pattern of events prevail in other dorylines, particularly in species which deviate noticeably from the type species in prominent aspects of behavior? *E. hamatum* is prominent in the forests of tropical America because it is a terrestrial species staging raids which involve far-flung systems of ramifying columns headed by small groups of predators, i.e., it may be called a column raider. Strikingly different are the swarm raiders, whose forays are headed by immense unitary bodies of ants rather than by innumerable small spearhead groups. It is typical in *E. burchelli*, which we have studied as a representative of the swarm raiders, that only one principal trail connects the primary raiding masses with the colony bivouac. Even so, *burchelli* colonies undergo a bivouac-change movement as sequel to a raid much as do *hamatum* colonies. There are radical differences between these species in the actual mechanism of the exodus as an outcome of raiding, yet we have found (Schneirla, 1944a) that certain fundamental events are common to both.

From this arises the principal subject of the present paper, the larger picture of relationships in the life and behavior of swarm-raiding *burchelli* colonies over considerable periods of time. If *burchelli* colonies undergo a cycle of movement and pause somewhat like that of *hamatum*, it is desirable to find how the phases of this cycle occur, and what their underlying causes may be. In that sense, the present investigation represents a test of the theory already advanced for the *Eciton* type species.

An interest in the causation of *Eciton* mass movements inevitably leads to questions concerning their relationship to the general occurrence of collective movement in animals. In the general literature this relationship is not at all clear, in particular because writers on animal movements have based their conclusions concerning the dorylines on doubtful and incomplete evidence.

In one of the most comprehensive treatises on the subject of animal movements, Heape (1931) distinguishes between two types of "voluntary" (i.e., active) movements: one, "elemental" or "climatic" movements, "undertaken in search of food or water, or in order to escape from unfavorable climatic conditions," and two, "gametic" movements, which are "impelled by impulses which arise from physiological activity in the reproductive system, and concern the life, that is to say, the continuity of the race" (p. 336). Heape insists that gametic (i.e., reproductive) causes are essential to true migration, ". . . a movement which involves a journey to a definite area, and a return journey to the area from whence the movement

arose." Distinguished from this is emigration, which rests upon alimentary or other non-gametic causes, and in which a return does not occur.

On this basis, Heape believed that doryline movements should be classed as non-migratory, as cases of emigration. For example, Eciton colonies, which "constantly change their home and hunting ground," are not accepted by him as valid instances of nomadism, since

"... these animals recognize no territory; they travel wherever it may suit them to go, establish themselves with their pupae wherever it may suit them to stay, and remain there as long as sufficient plunder is to be found within the limit of their raiding capacities. Moreover, they do not return to any permanently established home" (p. 178). "I judge them to be emigrants, whose sole business in life is raiding. They have a temporary home only, it is true, but still a home within a definite territory so long as it lasts; that is, so long as their ravages over that territory bring them in what they need. Then they move on somewhere, anywhere else, with their young; make there another home, and again ravage the territory available around."

This view of doryline movements, although misrepresentative (Schneirla, 1944a), appeared to find corroboration in the literature. From Carpenter's (1920) observations of day-long movements of *Dorylus* colonies in East Africa, "streaming along . . . to a new hunting ground, where they quickly form a home and establish themselves," Heape draws a distinction between such activities and "minor streams of hunting parties, which are formed as occasion demands after the colony has settled down in new territory." But, he says,

"while emigrating these ants do not carry on raids, they are concerned only in traveling to a new home. Presumably they have sufficiently divested the region around their last home, they have worked out that region, and now seek new territory to ravage, and being raiders, and living only by raiding, they disregard all territorial rights of others and establish themselves, where spoil promises to be plentiful."

This has been the prevalent view, that doryline colonies move from place to place rather haphazardly, purely on an alimentary basis. From his observations of Eciton behavior in Nicaragua, Belt (1874) stated that "... it is a curious analogy that, like the hunting races of mankind, they have to change their hunting grounds when one is exhausted, and move on to another" (p. 17). Vosseler (1905), having studied the doryline species *Anomma molestum* of East Africa, stated that a colony occupies the same nest until it has destroyed all of the available prey in a locality, which requires some 8 to 10 days, then the colony moves elsewhere. Once the vicinity of the nest has been thoroughly ransacked, when the haul of booty no longer suffices for the food needs of the colony, "so muss es sich zur Auswanderung entschliessen" (p. 293). Vosseler's account has led (or, we might say, "misled") numerous secondary writers, including Forel (1923), to adopt the food-exhaustion explanation of doryline movements. Reliance upon the presumed soundness of this interpretation brought Heape (1931) and Fraenkel (1932) in particular to the decision that Eciton movements are not to be considered instances of migration.

The last two writers, especially, might have been less positive concerning the status of Eciton movements had they not overlooked an important study by Müller (1886). Because this study is the only one preceding our own which contains information about the day to day activities of an Eciton colony (an *E. burchelli*

colony, moreover), and because it is published in a relatively inaccessible journal, it is desirable to offer here a detailed summary of the pertinent results.

Müller opened his record on Feb. 28, 1885, when a *burchelli* colony came to notice in his brother's garden near Blumenau, Prov. St. Catherina, Brazil, and ended about three weeks later. A summary of his observations follows:

Feb. 28 (on the two preceding days the ants had been seen raiding in the garden) at 9 A.M., when discovered on Feb. 28, the ants formed a closed column (very probably what we have termed the "principal column"—1940). Having viewed "all of the branches," Müller found headquarters beneath the branches of a fallen tree. Some of the larvae were enclosed in cocoons, but none had pupated. At 8:00 P.M. when the spot was revisited, a strong column of *Ecitons* was removing larvae with all signs of dispatch, moving in the reverse direction to that of the morning (i.e., away from the bivouac). At 11:00 P.M. the procession was still in full progress.

Mar. 1—The column was traced at 7:00 A.M. to a fallen hollow tree, into which the ants disappeared.<sup>2</sup> Since there were no traces of other columns from this site, which was about 200 paces from that of Feb. 28, Müller concluded that the hollow tree must contain the new nest.

Mar. 2.—At 6:00 A.M. the ants were engaged in changing quarters once more, this time having taken a fallen hollow tree-trunk 50 paces from the last one.<sup>3</sup> On this day also the ants went out pillaging, raiding to the east.

Mar. 3.—At 6:00 A.M. no ants were to be seen; a few hours later they were occupied with booty, on a trail to the southeast.

Mar. 5, 6—At 6:00 A.M. no ants were seen outside the nesting place.

Mar. 7—At 10:00 A.M. there was a single column, raiding almost directly westward. "Thus, in visiting the surrounding area on different days, the ants appeared to proceed fairly systematically."

Mar. 8—Despite good weather, the ants were not seen outside the tree when the site was visited at 7:30 and 11:00 A.M., and at 4:00 P.M.

Mar. 9—When no signs of activity were observed, smoke was introduced, forcing the ants to move into the lower portion of the trunk. No sexual forms were seen. All visible larvae appeared to be enclosed.

Mar. 10—The former place was deserted; finally the colony was found hanging in a cluster against the inner wall of a standing tree close by. (There is no mention of a raid.)

Mar. 11—No booty column was observed.

Mar. 12—When the site was visited toward evening, a meager raiding column was seen.

Mar. 13—The tree trunk was opened, and large numbers of cocoons were seen, but no fertile forms.

Mar. 14—The colony was anaesthetized and a considerable part of it, estimated at about 5,600 cu.cm. in volume, was taken away in a sack for examination. Less than half of the workers and about half of the brood were removed. Among hundreds of individuals examined, the brood contained only enclosed pupae, with the exception of a still un-pupated small worker.

Mar. 15—In a further exploration of the cluster in the hollow tree, masses of eggs were discovered.

Mar. 16—In the afternoon a weak column was observed, out for booty.

Mar. 17—The hollow tree was knocked over and the ants once more were driven out with smoke. A search failed to disclose the queen. This marked the end of the study.

In discussing his results, Müller focussed upon his observation of a decline in raiding after March 8, the day when (as he judged) the last larvae were enclosed.

<sup>2</sup> On the morning of March 1 there was observed at one side of the *burchelli* column a wingless male, half led and half pushed along by a few workers. This individual was accepted by a group of workers with whom he was placed, and hence must have belonged to the colony. Subsequent examination by Forel disclosed that this *burchelli* male actually was a *Labidus*, one of the group whose biological affiliations had been long in doubt.

<sup>3</sup> It is important to note that this was the last active bivouac-change movement of Müller's colony during the period of observation.

This parallel between decreased raiding and a change in brood condition seemed to him far from accidental, since "a colony needs less food once its brood is enclosed, the ants raid correspondingly less" at such times. Although this explanation of reduced raiding based upon an inferred reduction of food requirements appeared to be justified by the observed circumstances, two important points relevant to its validity were not considered in the discussion. These were, first, that important changes in brood condition must have occurred well before March 9, and second, that a considerable decrease in raiding must have taken place about March 2. Such facts lead us to a consideration of events beyond raiding, favoring a different explanation than the one Müller offered.

Other events in colony behavior such as the matter of changing the nest-site appear to have received a very separate consideration in Müller's thinking. To him "the helplessness, the difficulty, with which they settled upon a change of dwelling after March 9," in contrast to earlier changes of quarters, could be explained only by assuming that a fertile queen was present (i.e., after March 9). Heavy and difficult to transport, he thought, this individual must have been a hindrance to removals. Although she must have been present on Feb. 28, the queen then very probably was "slighter and more easily transported." A direct contradiction of this line of reasoning exists in Müller's own evidence, in the fact that the colony kept to the spot even after the queen apparently had laid her eggs and might therefore have been "easily transported." To add to the difficulty, his own record shows that the colony first settled in place not on March 9, but actually on March 2. The facts call for a very different type of explanation.

Thus Müller advanced two distinctly different hypotheses, one to account for changes in raiding behavior, the other to explain colony immobility. The first of these is significant, not because it is valid as an explanation, but because it suggests that a relationship may exist between colony behavior and observable conditions within the colony. It was with lively interest that the present writer first read Müller's paper after having prepared an account of his own initial Eciton investigation (1933) in which the existence of such a relationship was noted and its significance indicated.

A final statement deserves to be quoted:

"Wenn nun das Vorhandensein einer Königin ein Hindernis für das Weiterwandern, die Zeit der Puppenruhe die Zeit eines viel geringeren Nahrungsbedürfnis ist, bei dem das sonst in wenigen Tagen abgesuchte Terrain wohl Wochen lang genügt, dann erscheint dies zeitliche Zusammenfallen zweier nicht in direkten Zusammenhang stehender Ereignisse (Verpuppung und Beginn der Eiablage) nicht als etwas Zufälliges, sondern als durchaus notwendig."

This suggests that Müller glimpsed the unity of the entire phenomenon, although the fixity of his two disparate hypotheses prevented him from reaching a common basis of theoretical explanation.

One relationship in the complex Eciton pattern about which a clear understanding is essential concerns the bearing of raiding activities upon colony movement. Of course, in the form of the behavior and in the time of day at which these two activities occur there are important typical differences, as a number of writers (Bates, 1863; Müller, 1886; Carpenter, 1920) have pointed out for various dorylines. From his observations of the African species *Dorylus nigricans* and *glabratus* Carpenter (1920) distinguished raiding forays from other movements. "But

every column of *Ensanafu* that one sees is not engaged in changing camp; most often they are either going out to hunt, or returning laden with spoils." We have shown in detail for *burchelli* (1944a) that raiding is organized in the morning and develops during the day, whereas the bivouac-change movement occurs at the end of day and during the night. Yet to differentiate raiding from colony movement should not lead to overlooking the relationship of these events, as Müller did in offering distinctly different explanations for them.

As a foundation for the present paper, our previous consideration (1944a) of the manner in which raiding leads into colony movement under given conditions is materially important. For in that connection it was shown that under certain other conditions a raid *cannot* eventuate in a change of nest site. The solution lies in the fact that very different conditions exist within the colony at the two times, as we have demonstrated in the case of the column-raiding *E. hamatum* (1938; 1944b). Although the form of *burchelli* raiding differs materially from that of *hamatum* forays, the fact that in both species colony movement occurs as the outcome of raiding suggests the existence of further similarities.

The purpose here is not merely to compare the nomadic activities of two *Eciton* species, although this is a useful way to extend our explanation of the *Eciton* problem as such. Beyond that, we are led to an interest in how *Eciton* movements fit into the general picture of the mass movements of animals. It will be useful to consider whether the *Ecitons* are truly migrant, in the sense that they may possess the most essential features of animal migration in general, or whether certain criteria generally considered essential to migration proper are lacking in these insects.

This study was conducted at the Barro Colorado Island biological reservation in Gatun Lake, Panama Canal Zone. The records were obtained during the rainy months May to September, for the most part during the years 1936 and 1938. The method was largely one of field observation, supplemented by special observations and tests in the laboratory. The general plan was to keep certain colonies on record over a considerable period of time, in order to study changes in their external behavior and internal condition during the interval; and correlatively, to obtain records of similar events in other colonies over relatively short periods of two or more days.

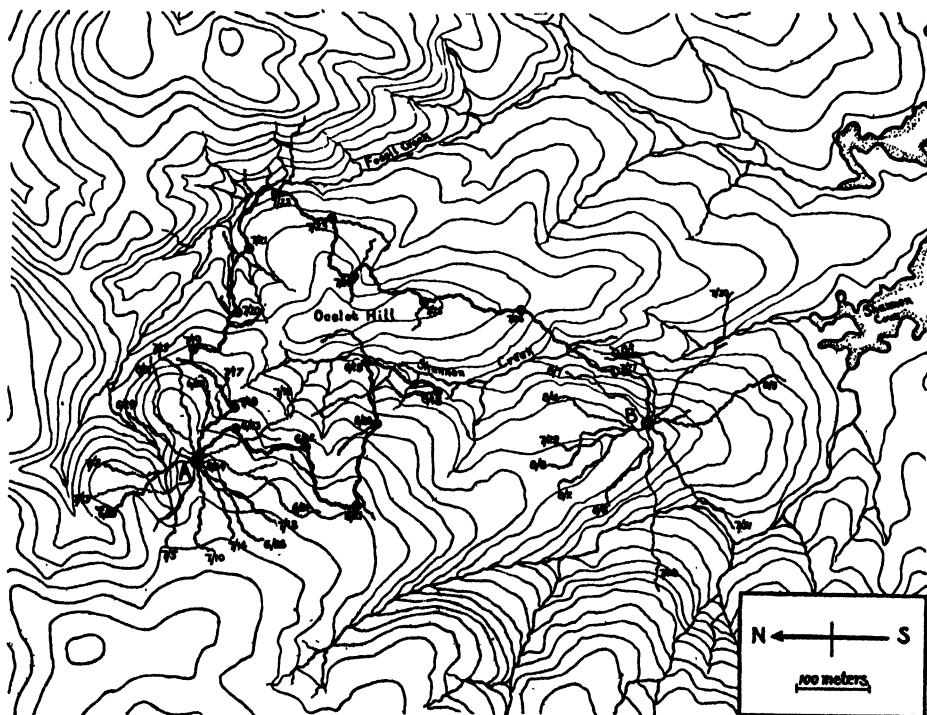
## RESULTS :

*Activities of a representative colony of E. burchelli:* This colony of *E. burchelli* (record number 38I) was first encountered on the morning of June 18, 1938, when it was engaged in a vigorous swarm raid. That evening it moved to a new bivouac site, and subsequently the study of this colony became the chief problem of the daily routine. The period of study ended on August 10 with the writer's departure from the Island, a total of 53 days in which colony 38I was under daily observation. During that time the colony moved over rugged terrain through forest a distance of nearly two miles, in an irregular path.

The manner in which the colony operated during the period of study is sketched in Figure 1, in which are represented the various bivouac sites successively occupied, the principal lines of most of the daily raids, and the routes of successive bivouac-change movements. The principal facts concerning the activities of colony 38I are given in Table I. The following discussion of this record is divided

into sections according to certain major changes which occurred successively in the behavior and condition of the colony.

**Period 1 (nomadic):** When the study opened, for a few days the colony staged very large daily raids, each passing over toward evening into an exodus which ended sometime during the night after the entire colony had moved to a new bivouac site. Activities began promptly each morning at daylight with the divergent spreading of ants around the bivouac, and matters progressed so that a directionalized swarm was formed within less than two hours. The swarms that developed commonly exceeded 10 meters in width by 11:00 A.M., and became somewhat unwieldy and disorganized about that time, so that one or more divisions subsequently occurred resulting in two or more sub-swarms moving divergently.



The outcome was a complex raiding system to be observed in the afternoon, with sub-swarms operating more or less independently although interconnected in the rear by various secondary columns, the latter discharging into a principal column communicating with the bivouac. The forays developing on the first six days of this study, up to and including June 23, conformed essentially to the pattern of "maximal" *burchelli* raiding previously described.<sup>4</sup>

All six of the bivouac clusters formed successively in this brief period were lo-

<sup>4</sup> The descriptive statements of this section concerning the development of a raid in *E. burchelli* are consistent with the results of a detailed analytical study of swarm raiding previously reported (Schneirla, 1940).

cated in the open and close to the ground. Typically the colony formed a cylinder which hung down about 40 cm. to the ground from where ants in the upper fringes of the wall were securely hooked by their tarsal claws to the lower surface of a log or mass of vines. Two of the bivouacs were pouches formed against trees one or two meters from the ground.

Generally in the middle of the afternoon or if delayed, by dusk, a large-scale exodus was recognizable, as a rule monopolizing the route of the principal raiding trail. In three of the six instances this exodus during the afternoon was blocked from the principal trail by return traffic, and developed a new route eventually followed by the colony movement. The rise of a bivouac-change movement as an outcome of burchelli raiding has been described in some detail (Schneirla, 1940, 1944a).

The transfer generally was completed before midnight, except as on June 23 when afternoon traffic difficulties delayed the beginning of the heavy exodus until early evening.

The terminal removal of the series took the colony to a hollow tree only about 45 meters from its previous bivouac site. Within this tree it clustered at a considerable distance from the ground. From other observations it can be said that this last movement of a nomadic series generally is distinguished from preceding ones, as in this instance, by the relative shortness of the trek as well as by the nature of the bivouac site. Under these conditions the colony tends to cluster well above the ground, commonly within a hollow tree or similar secluded situation.

The queen was not seen during this series of moves. In each of four instances when the queen of another burchelli colony has been observed under conditions comparable to these, this individual was well contracted and (appearing in the last third of the movement) was able to force a way despite the crowding of excited workers around her.

**Period 2 (statory):** On the night of June 24 the colony formed an elongated pouchlike cluster high (ca. 10 meters up) in the interior of a large lightning-cleft tree. The bivouac remained in almost exactly the original position (where it was well sheltered from the elements) until July 16, i.e., for a period of slightly more than three weeks.

During this interval of immobility raids when they occurred were definitely smaller than before June 24, fitting the picture of "minimal" raiding previously described for burchelli (1944a). The forays were always slow to begin in the morning (although before July 24 activities began at dawn) and occasionally did not begin until afternoon. On some of the days, as Müller observed in his colony, there was no raiding at all. This interval of frequent raidless days began about one week after the time of the last movement, roughly in the middle of the period. When raids occurred they were relatively small, without major swarm divisions such as those commonly observed in nomadic burchelli colonies.

In the early afternoon of each raiding day there occurred a recognizable resurgence of ants from the bivouac into the raiding system, although this afternoon exodus was definitely weaker than in raids preceding June 24. Also, a more or less extensive return to the bivouac typically was under way at dusk. After July 13, when the raids increased noticeably in strength, the return to the bivouac frequently was observable until after midnight.

**TABLE I**  
**Changes in the activity and condition of *E. burchelli* colony 38I between June 18 and August 10, 1938 (cf. Figure 1)**

| Date    | Bivouac  | Condition of the brood                           | Raiding activity  | Behavior toward end of day  |
|---------|--|--|---|---|
| June 18 | A cylinder hanging from brush to ground.                                     | Single brood; moderately developed larvae.       | A well-developed foray to the <i>N</i> , with swarm division. New push from bivouac in afternoon. | Somewhat delayed bivouac-change, over new route to <i>N, NE</i> started in afternoon.   |
| June 19 | A flat low cylinder beneath a log.   | Single brood; moderately developed larvae.       | A very active raid to <i>NE</i> $\times$ <i>N</i> ; three swarm divisions.                        | Similar to above; movement to <i>S, SW</i> over route started in afternoon.             |
| June 20 | Long mass pouched against tree, one meter from ground.                       | Single brood; moderately developed larvae.       | A vigorous raid to <i>NW</i> ; two swarm divisions.   | Movement to <i>W, NW</i> over raiding trail established in morning.                     |
| June 21 | A long, bulbous mass suspended in vines, one meter from ground.              | Single brood; moderately developed larvae.       | Very large raid to <i>N, NE</i> , with swarm divisions.   | Movement delayed until evening, occurred over raiding route to <i>N</i> and <i>NE</i> . |
| June 22 | A long cluster from brush to ground.   | Single brood; moderately developed larvae.       | Very active raid to <i>N</i> ; swarm divided three times.   | Moved to <i>N</i> in late afternoon over raiding route.                                 |
| June 23 | Bulb-shaped mass suspended between buttressed tree roots.                    | Larvae close to maturity. Spinning observed.     | Maximal raid to <i>NW</i> ; swarm divided first at 9:30 A.M.                                      | Short distance to <i>NW</i> over raiding route; delayed until evening.                  |
| June 24 | Site A: A large pouch against inside wall of hollow tree, about 8 meters up. | Larval cocoon-spinning in full swing during day. | Moderately active raid to <i>S, SW</i> ; definitely less active than on previous days.            | No movement. All raiders had returned to bivouac before 10:00 P.M.                      |
| June 25 | Site A: no change.   | Cocoon-spinning observed, but less widespread.   | Moderate raid to <i>S, SW</i> ; no swarm division.  | No movement; return traffic dominated principal trail after 2:00 P.M.                   |
| June 26 | Site A.  | Spinning less noticeable.                        | Moderate raid to <i>S, SW</i> ; no swarm division.  | No movement; raiders still returning at 8:30 P.M.                                       |
| June 27 | Site A: cluster somewhat smaller   | Spinning of outer envelopes evidently completed. | Less active raid to <i>NE</i> ; no swarm division.  | No movement; nearly all raiders returned before 8:00 P.M.                               |
| June 28 | Site A.  | Enclosed brood, still spinning inside envelopes. | Raid to <i>E</i> ; small swarm, no division.  | No movement; general movement toward bivouac at 5:00 P.M.                               |
| June 29 | Site   |  | Minimal raid to <i>N, NE</i> .  | No movement; general return at 6:00 P.M.  |
| June 30 | Site A.  | Brood pupating.                                  | Not raiding at 10:30 A.M., possibly no raiding at all.  | No movement. Not visited in afternoon or evening.                                       |
| July 1  | Site A.  |  | Not visited.  |   |
| July 2  | Site A.  | Brood pupating.                                  | Small raid to <i>NE, N</i> .  | Return to bivouac complete by 9:45 P.M.   |
| July 3  | Site A.  |  | Not visited.  |   |
| July 4  | Site A.  | Brood all pupated. New batch of eggs present     | No raiding.   |   |
| July 5  | Site A.  | Brood all pupated. New batch of eggs present.    | Moderate raid to <i>NW, W</i> .   | General return in progress at 2:00 P.M.   |
| July 6  | Site A.  |  | Not visited.  |   |
| July 7  | Site A.  | Brood all pupated. New batch of eggs present.    | No raiding.   |   |
| July 8  | Site A.  | Brood all pupated. New batch of eggs present.    | No raiding at 10:00 A.M.  |   |
| July 9  | Site A.  | Brood all pupated. New batch of eggs present.    | No raiding.   |   |
| July 10 | Site A.  | Enclosed broods; hatched eggs seen.              | Moderate raid to <i>W</i> .   | General return in progress at 4:30 P.M.   |
| July 11 | Site A.  | A small number of cocoons opened.                | No raiding.   |   |
| July 12 | Site A.  | A few more cocoons opened.                       | Moderate raid to <i>S, SW</i> ; no swarm division.  | Raiding ceased before dusk; general return during early evening.                        |
| July 13 | Site A.  | Further cocoon-opening.                          | Moderate raid to <i>NW</i> ; no swarm division.   | General return completed before 10:00 P.M.  |
| July 14 | Site A.  | Cocoon-opening more active; continues at night.  | Moderate raid toward <i>SW</i> ; no swarm division.   | General return completed about 11:00 P.M.   |
| July 15 | Site A: cluster appears larger.  | Cocoon-opening apparently increasing.            | Moderate raid toward <i>NE, E</i> .   | General return observed at 7:00 P.M.  |
| July 16 | Site A: cluster much larger.   | Cocoon-opening at its peak.                      | Raiding to <i>S, SE</i> , noticeably more vigorous.   | General return still under way at 11:00 P.M.  |

**TABLE I—Continued**  
**Changes in the activity and condition of *E. burchelli* colony 38I between June 18 and August 10, 1938 (cf. Figure 1)**

| Date    | Bivouac  | Condition of the brood  | Raiding activity   | Behavior toward end of day  |
|---------|--|---|--|---|
| July 17 | Site A: cluster still large.   | Cocoon-opening still in active progress; callows on trail near bivouac.               | Vigorous raid to SE, E, with swarm division.   | Bivouac-change movement to SE, E over raiding trail, well under way at 4:00 P.M.                                |
| July 18 | New bivouac, a cylinder hanging to ground from root.                   | Last cocoons opened; callows seen on raiding trails; brood of very small larvae seen. | Maximal raid to NE, with two divisions of swarm.   | Movement to NE under way at 3:40 P.M., over raiding route.  |
| July 19 | A cylinder beneath raised tree roots.                                  | Callows seen farther out on raiding trails.   | Maximal raid to S, SE; swarm division at 10:30 A.M.  | Movement to SE over raiding trail, under way at 6:00 P.M.   |
| July 20 | Cylinder hanging from vines to ground.                                 | Callows now common on raiding trails.   | Maximal raid to E; swarm divides twice.  | Movement to E over raiding trail, under way at 4:45 P.M.  |
| July 21 | Two cylinders hanging from log to ground.                              | Larvae now observed in act of feeding.  | Maximal raid to E; one major; one minor division.  | Movement to SE, E under way at 3:30 P.M.; greatly delayed by marginal "traffic jam."                            |
| July 22 | Cylinder hanging from raised roots to ground.                          | Callows seen near base of swarm.  | Maximal raid to SE, E; swarm divides early; new push from bivouac in afternoon.                      | Movement to SW, S over raiding route of new afternoon push.   |
| July 23 | Bulb formed around small tree 2 meters from ground.                    | Larvae now approach full growth; callows numerous in swarm.                           | Maximal raid to SW, S; swarm divides early, at 9:00 A.M., twice later. New push in afternoon.        | Movement delayed by traffic jam near bivouac, under way at 7:00 P.M., over new trail of afternoon to W, SW.     |
| July 24 | A cylinder between roots, resting on ground.                           | Large larvae now well distributed through bivouac.                                    | Maximal raid to SE, E and S; first swarm division at 8:30 A.M.; new push to W begins about 3:00 P.M. | Movement delayed by first exodus to W; finally under way to SE X S at 8:00 P.M.                                 |
| July 25 | Clustered inside open end of hollow log.                               | Larvae nearing full maturity; a few instances of spinning.                            | Vigorous raid to S; two or more swarm divisions.   | Movement delayed by return traffic; under way to S at 6:30 P.M.   |
| July 26 | Bulb formed against tree one meter from ground.                        | Larvae widely distributed through cluster. More spinning.                             | Maximal raid to S, SW; major swarm division at 9:00 A.M., two others later.                          | Movement delayed by traffic jam at outer junction; under way to S, SW at 6:00 P.M. Bivouac forming at 7:00 P.M. |
| July 27 | Long cluster one meter from ground, around tilted tree.                | Larvae widely engaged in spinning.  | Maximal raid to S, then turns westward.  | Movement over raiding trail to S, then W; delayed by traffic difficulties.                                      |
| July 28 | Site B: in upper interior of hollow tree.                              | Cocoon-spinning visible on shelf in tree interior.                                    | Moderate raid on continuation of 7/27 route to W.  | No movement; general return after raiding ceased at dusk.   |
| July 29 | Site B.  | Brood subsequently inaccessible.  | Less active raid to NW, W.   | No movement.  |
| July 30 | Site B.  |   | Moderate raid to SE.   | No movement.  |
| July 31 | Site B.  |   | Moderate raid to SE.   | No movement.  |
| Aug. 1  | Site B.  |   | Moderate raid to E, then bends to N; no swarm division.  |   |
| Aug. 2  | Site B.  |   | Less active raid to NW.  | No movement.  |
| Aug. 3  | Site B.  |   | No signs of raiding at 4:00 P.M.; probably no raiding at all.  | No movement.  |
| Aug. 4  | Site B.  |   | Minimal raid to NE X N.  | No movement.  |
| Aug. 5  | Site B.  |   | No raiding.  | No movement.  |
| Aug. 6  | Site B.  |   | No raiding until 2:00 P.M., then raid started to NW.   | No movement.  |
| Aug. 7  | Site B.  |   | Moderate raid to N, NE.  | No movement; general return in progress at 6:00 P.M.  |
| Aug. 8  | Site B.  |   | Moderate raid to N, then NW.   | No movement.  |
| Aug. 9  | Site B.  |   | No sign of raiding at 2:30 P.M.; raid started after 3:00 P.M. to S, SE.                              |   |
| Aug. 10 | Site B; probably occupied for another week. Observations discontinued. |   | Minimal raiding to SE, stopped by protracted rain at 3:00 P.M.                                       | General return slowly under way after 6:00 P.M.   |

The colony remained at site *A* in the hollow tree from June 24 to July 17, an interval of 23 days. During this interval 15 daytime forays were observed. Each of these raids, as may be seen in Figure 1, kept roughly to a given general direction, a characteristic of *burchelli* raids previously discussed (1940).

When we measure the angle of divergence between the main line of each raid and that of the following raid (excepting the three instances when the colony was not visited), the values with one exception are found to lie between  $30^\circ$  and  $160^\circ$ .<sup>5</sup> Thus, although there was no particular order in the directions of successive raids, evidently there were factors opposing two raids in close succession into the same terrain. It is apparent from the figure that the raids were scattered fairly well in all directions around the bivouac. Such is not always the case, especially when vegetation and terrain present differences around the bivouac which may affect distribution of prey or differently canalize the first spreading of the ants in the morning. Not all instances support, as well as did this one, Müller's impression that the environment is probed in a thorough manner.

However, it must not be thought that even during a stop of 23 days at site *A* the raids of this colony even came close to depleting the supply of prey in the surrounding area. The record shows that large sectors, and even certain zones fairly close to the bivouac, were covered incompletely or not visited at all. Moreover, areas cleared of prey may be speedily repopulated. Three days after colony 38I had moved from this site, another *burchelli* colony staged a vigorous raid fairly close to the bivouac tree on the west, with evident signs of a large haul of booty.

**Period 3 (nomadic):** On July 18 there occurred a foray which clearly surpassed in vigor and numbers of participants any raid since June 24, and which ended in a bivouac-change movement along the principal raiding trail. The exodus led to a marginal site established in the evening, more than 100 meters from the long-tenanted one in the hollow tree. Thereafter the bivouac site was changed daily, as described for period one above. All clusters were formed in exposed places.

A difference worth mentioning is that whereas the bases of all of the first five bivouacs touched the ground, three of the last five clusters were in somewhat elevated positions. On July 17 in the evening the ants began a cylinder on the ground between tree roots, only to desert this for an elevated position early in the night. All raids of this period were vigorous or "maximal" ones, in which activities began promptly at dawn and one or more swarm divisions were the rule. Since the ants seldom worked back through the approach-area of the preceding day for more than two or three hours in the early morning, the major new raiding push always developed into unworked terrain in some other direction.

This interval of maximal raiding and daily bivouac-change ended on July 27, after eleven days on the march.

**Period 4 (statory):** A vigorous raid on July 27 terminated in a delayed night movement, relatively short in comparison with preceding movements, which took the colony to a clustering place high (evidently 15 meters or more aloft) in the upper interior of a huge lightning-cleft tree. Unfortunately the cluster lay out of sight in the dark upper interior of the tree, where it could not be illuminated or reached in any feasible way.

During the last three days of the study measures of desperation such as dust shot were used in attempts to puncture the bivouac and obtain samples of the

<sup>5</sup> The exception was the raid of July 18, which started from the bivouac on the trunkline developed on the previous day and used about 60 meters of that route; however the extension of this raid after mid-morning diverged about  $35^\circ$  from the main direction of July 17. Incidentally, the resumed use of a previous principal trail by a statory colony is not infrequent in *E. hamatum*, although it is relatively rare in *burchelli*.

brood, without success. When raids occurred, the ants reached the ground as a rule in one or more broad columns on the outside of the tree, traceable from the ground to a height of nearly 20 meters before visibility became uncertain.

Although at first the raids occurred daily and were moderately developed, after five days there were intermittent days without any sign of raiding, or with limited afternoon forays. From the first day at site *B* morning activities began slowly and sluggishly; later on some of the raidless days the ants swarmed down before mid-morning only to withdraw without having begun a foray on the ground. (It is of course possible that unobserved foraging occurred in the upper section of the bivouac tree.) When raids occurred, a general return to the bivouac uniformly began as a persistent movement during the afternoon and was always well under way by dusk. With the exception of ants in procession evacuating the raiding system of the day, no activity of the colony was observed outside the bivouac-tree at night.

When the study had to be discontinued the colony had been at site *B* for 13 days. (Very probably it remained several days longer.) During this time it staged eleven raids, only three of them within the southeast and southwest quadrants.

At this site, as may be seen in Figure 1, the raids were much more concentrated within given sectors than within others. Numerous factors were evidently involved. Downhill at a distance on the south lay a stream nearly impassable to the burchelli, also in beginning a raid the burchelli masses frequently were deflected from that side by the narrow columns of a hamatum colony at the time in statary bivouac near the stream. Uphill toward the north, along a 30° slope, the going in general was easier; then too, the burchelli columns generally reached the ground on that side of the bivouac tree.

The successive raids were well differentiated in direction, with the smallest angle of difference between the lines of successive raids 50°, the largest 165°. Except for an occasional overlapping close to the bivouac, no two successive raids followed the same route, a result not attributable to chance.

*Condition of successive broods in colony 38I:* When it came under observation colony 38I had a single brood (estimated in the tens of thousands) in the advanced larval condition. A thorough examination of the bivouac on June 20 (four days before the colony stopped moving) disclosed no other broods. In the bivouac the larvae were distributed widely throughout the mass; many were heaped in interior pockets, and large numbers were held individually by ants in the outer wall of the cluster. During bivouac-change movements the larvae were carried individually, gripped anteriorly in the carrier's mandibles and slung underneath her body in the typical Eciton manner. In general, larvae were transported in the latter half of the movement.

Although the beginning of cocoons by mature larvae in this brood was first observed on June 23, it may have begun shortly before that time, since three of 40 larvae taken to the laboratory were seen in the act of spinning on June 22. On June 24, when the colony began its long-term residence in the hollow tree at site *A*, spinning was in progress as a general activity. Larvae were then spread about over the detritus flooring the tree hollow, with a constant interpassage of larva-carrying adults between the cluster above and this spinning area below, as described for burchelli by Beebe (1919). The process of forming the outer envelopes appeared to have been finished before June 27, since all larvae that fell to the ground

when the bivouac was punctured were enclosed, and all of the laboratory lot of larvae were covered by that time. This does not mean that the larvae had become immobile. Movements of enclosed larvae within their tissue envelopes were observed up to June 28, indicating further spinning through which cocoon walls were thickened. It can be said that colony 381 entered the statary condition rather abruptly on the day when the major part of its larval brood became enclosed.

This brood pupated and passed through its pupal stage while the colony was stationed at site *A*. The removal of callows from cocoons (by workers) began on July 11 and continued on following days, although at first the activity was limited, judging by the fact that only a few dozen empty cases fell from the bivouac on any one day before July 14. Gradually thereafter the removal of callows continued at a sharply increasing rate, indicated not only by a considerable increase in the number of empty cases on the ground and interior wall of the tree below the cluster, but also by a great increase in cocoon-opening visible at the surface of the bivouac. This activity appeared to reach its peak on the night of July 16 and during the day on July 17. On July 17 the colony staged a great raid and moved from the site at the day's end. The bivouac-change column that night was thronged with callow workers. Cocoon-opening evidently was nearly completed before the move, since it was estimated that only a few hundred cocoons were carried to the new site, where the last callows were removed on July 18.

Thus the time when nomadic activity was resumed by this colony coincided exactly with the appearance of a mature brood as newly delivered callows. At first these young workers remained at the bivouac except during bivouac-change movements; however during the first week after leaving site *A* they were observed at points in the raiding system successively farther from the bivouac, and on the sixth day (July 23) they were numerous in the raiding swarm. At that time pigmentation had increased so that they had nearly the color and dusky appearance of adult workers.

A further brood in the early larval stage was found on July 18, when the first nomadic bivouac was examined. In a sample of 82 larvae taken on the evening of July 24, body lengths ranged between 2.4 and 6.3 mm. At that time, therefore, this larval brood was well advanced in growth.<sup>6</sup> Since the colony had only a brood of mature larvae when it came to site *A*, this new brood must have hatched from eggs laid during the stay in the hollow tree. The queen, observed in the latter section of the July 19 bivouac-change column, was fully contracted. Her condition evidently did not change during this period of daily movement, since no eggs were found in periodic examinations of the bivouac and the queen was still fully contracted when seen on the night of July 26.

The new brood, in the early larval condition when the new nomadic period began, advanced through its larval development during the following days of colony movement. On July 25 scattered instances of spinning were observed, and on July 27, with the colony clustered around a small inclined tree well away from the ground, spinning activity was well under way. All circumstances indicated that when the colony settled next day within the hollow tree at site *B*, the bulk of

<sup>6</sup> This may be compared with a sample of 700 larvae taken from another *burchelli* colony on the first day of a nomadic phase, when body lengths ranged between 0.32 and 1.9 mm. (skewed as usual toward the smaller sizes).

its mature larval population was engaged in spinning or already provided with an outer envelope.

A parallelism between colony behavior and brood condition during the 53 days of this study is clearly indicated in the findings. In particular, it will be noticed that the behavior of the colony and the status of its brood were in substantially the same relationship at the end of the second nomadic period (i.e., on July 27) that they held at the close of the first period (i.e., on June 23). In both cases the colony ceased maximal raiding and bivouacked in a secluded place when its mature larval brood had reached the peak of its relatively brief spinning episode. If the study could have continued about one week after August 10, without much doubt the second statary period (i.e., at site *B*) would have been observed to end as did the first one, with the emergence of a mature brood as callow workers.

It is regrettable that the time when the new batch of eggs was laid could not be ascertained during either statary period. Circumstantially we know that eggs were laid at some time during the stay at site *A*, and unless the statary period at site *B* was very exceptional, we may surmise that the same event occurred there. Direct facts concerning the oviposition were not obtained in either case, since at site *A* the colony was beyond close examination and at *B* was entirely out of reach. Fortunately, observations on other burchelli colonies over shorter periods of time are available which indicate that in the rainy season a batch of eggs is laid early in each statary period, and which fully corroborate the description of a brood-colony behavior parallelism given above.

*Observations paralleling the study of colony 38I:* On July 18, when colony 38I was beginning an interval of nomadic activity, a burchelli colony was discovered in the southwest section of the Island, sequestered in the crown of a tall palm tree. Although the bivouac cluster itself was never seen, its position was approximated by noting where columns emerged to descend the trunk in the raids of succeeding days. This colony remained on record until August 6. During these 18 days ten raids were observed, probing successively in various directions, all of these raids relatively small and slow to begin in the morning. In the nine days after July 20 there were four days when no raiding occurred, although forays were staged on each of the last six days of the colony's stay in the palm tree. The colony moved to a new site on the evening of August 6, and a further move was observed on the following evening. Thousands of callow workers crowded the columns, most of them very recently emerged as indicated by their limitations of movement and pale pigmentation. Unopened cocoons were transported in the column; however their limited number in comparison with the hordes of callows indicated that the great majority of the mature brood had been removed before the march began. In addition, a very young larval brood was present, carried in several small packets by ants in the column. (In a sample of 50 larvae, the body lengths ranged from 0.3 to 1.8 mm.)

These circumstances suggest that the bivouac-change movement marked the end of a statary period of 18 + days in the palm tree, during which a pupal brood matured and a further brood passed through its early larval development. In addition to the typical occurrence of raidless days, it should be noted that raids occurred on all of the last six days at the site. A fact of interest is that this colony remained in place throughout the 11-day nomadic interval of colony 38I, and took to the march six days after 38I had stopped at site *B*.

A different periodicity was involved in another colony for which the onset of the statary period was observed. On July 24 (i.e., just four days before colony 38I reached site *B*) this colony was engaged in a large raid about one-fourth mile east of the laboratory clearing. That night it moved about 120 meters to a new bivouac site, and on the following day a large raid gave rise to a move (a short one) to a new site south of the clearing. This time the colony clustered about four meters from the ground, within an exposed hollow in the trunk of a large tree. On the evening of July 25 and on the following day extensive spinning activity was observed, with heavy columns of workers carrying larvae between the bivouac cluster and a debris-strewn place in the cavity near the ground. Here larvae were spread around in the wood-dust and were constantly being shifted in position by workers (cf. Beebe, 1919). On the morning of July 27 a general reduction of activities in and near the bivouac indicated that the major episode of spinning had terminated. The bivouac then assumed a regular pouch-like form which was changed only in minor ways during the following two weeks.

The colony was still bivouacked at this site on August 10, after it had passed 17 days there in the statary condition. During the first week of the stay, there were daily raids of only moderate vigor. Later, in the last ten days of the observation, forays became less active and less frequent than before,—three raidless days were noted, and very probably there were others. Because of the position of the cluster its contents could not be examined closely, hence the appearance of a possible new brood could not be ascertained.

It will be noted that this colony began its statary period just two days before colony 38I stopped at site *B*, and that both colonies remained in place when the investigation stopped. The externally observable circumstances were much the same in the two cases, especially the concurrence of the final halt with principal enclosure of the mature larval brood and with a marked reduction of raiding from the nomadic level. Numerous studies of shorter duration with other colonies fully confirm these observations.

One important fact should be added here. Among the colonies for which the beginning of the statary period was recorded, in three instances the bivouacs were accessible to direct examination of their contents. From the study of these colonies during the first two weeks of the interval it can be said that a new batch of eggs appears at some time between the sixth and the fourteenth day. Considering the fact that broods of very young larvae at closely comparable stages of development are found in the colonies just beginning a nomadic period, the above observations may be taken to indicate approximately the typical starting time of the new brood in *burchelli*.

*An experimentally modified burchelli routine:* On June 17, 1933, a *burchelli* colony was discovered (on Donato Hill) in a pouch bivouac suspended from crossed vines against the side of a large tree, mainly within a deep recess between buttressed roots and behind large vine stems, about three meters from the ground. The colony remained in this situation until June 26, despite being disturbed on three separate occasions when the bivouac was opened for examination of its contents. At best the raids were only moderately developed, without a major swarm division. During the first five days of the study there were two raidless days, and on another day the foray began in the afternoon. Then in the next five days, just preceding a change to nomadism, daily raids occurred.

When the study opened, the colony possessed a fairly advanced pupal brood, entirely enclosed, and some large packets of eggs were also present. On June 21 and 22 there were indications of cocoon opening; on the afternoon of June 24 removal of callows was actively in progress, and on June 25 when a vigorous raid occurred the removal of callows seemed to reach its peak. That night the colony moved, leaving a litter of pupa cases on the ground below the vacated bivouac site.

However, the colony left the site with only a fraction of its callow brood. On June 23, using ether, I collected an estimated three-fourths of the advanced brood, mainly advanced pupae still enclosed, with a minority of free callows, together with a small part of the larval brood. A subsequent census of the material gives a total of 31,298 individuals in cocoons, all far advanced in pupation.<sup>7</sup>

To find whether there were any polymorphic differences in developmental point attained the cocoons were sorted according to size and then were separated into two groups: one, well pigmented and two, little pigmentation. (Pigmentation here may be taken as a rough indication of nearly complete maturity.) It was found that only 2 per cent of the smallest individuals (median length, 5.0 mm.) had much pigmentation, whereas 50 per cent of the next larger group (6.5 mm.), 89 per cent of the next larger (7.5 mm.) and virtually all individuals in the largest sizes (median lengths, 9.0 and 10.2 mm.) were well pigmented. This shows that the greatest number of pupae furthest advanced in development tends to be in the larger polymorphic categories.

In these facts, together with our observation that ordinarily only well-pigmented individuals (i.e., those capable of movement) are removed from their cases by adult workers, we find in these facts confirmation of Müller's impression that workers major and the larger sizes in general tend to emerge as callows in advance on the smaller individuals. Our observations on the responses of adult workers to the nearly mature brood (1934) show why the larger individuals, those more advanced in development, are the ones to be removed first from their cocoons. After June 23 it was observed that antennal and leg reflexes were common among the best pigmented individuals, and many of these callows survived when artificially removed from their cocoons. Direct observations in the laboratory yielded further evidence that the opening of cocoons by workers depends to a great extent on the responses of adults to the movements of mature pupae within the cocoons, a fact previously reported for *E. hamatum* (Schneirla, 1934).

In the following days the behavior of this colony was exceptional, in that it seemed definitely sluggish and reduced in comparison with an ordinary colony in the interval following the appearance of a callow brood. Throughout the week following the first bivouac-change movement, although daily raids occurred these were inferior in vigor and numbers to maximal *burchelli* forays, and frequently a daily raid was not followed by a change of bivouac.

On the second nomadic day (June 26) the colony moved, but covered a relatively short distance of 40 meters; on the third day (June 27) there was no colony movement, and although the bivouac was changed on the evening of June 28 this too was over a relatively short distance of 25 meters. On June 29 (when callows were first observed in the swarm), a raid to the west ended in a general return to the bivouac of the day, a cylinder extending to the ground from matted vines. Similarly on June 30 a raid toward the northeast was only moderately developed, and at 4:00 P.M. a general return to the same bivouac was well under way. On July 1 a raid to the southeast ended in a short bivouac change of about 30 meters. However, the move on the evening of July 2 took the ants about 70 meters to the southeast, and that of July 3 eastward about 60 meters where they clustered within

<sup>7</sup> The results of the census were obtained mainly through the capable assistance of Mr. Frank Trainor, whose services were made available through the Gibson Committee and W.P.A.

TABLE II

Summary of principal changes in the condition and behavior of an *E. burchelli* colony on record from June 17 to July 25, 1933

| Period                            | Bivouac   | General nature of raiding   | Occurrence of colony movement   | Condition of brood   |
|-----------------------------------|---|---|---|--|
| I<br>June 17 to June 26 (statory) | Pouched against side of tree, well above surface of ground.                           | In general, minimal, with no forays on certain of the days; increasing activity near end of the period. | No bivouac-changes. Returned to same clustering place at end of each day's raiding            | Enclosed pupal brood, emerged from cocoons at end of period; three-fourths of this removed. New brood of eggs and young larvae found 6/18. |
| II<br>June 27 to July 7 (nomadic) | Generally a cylinder hanging from low object to ground; less often a suspended pouch. | Only moderately active at first; maximal raids in latter part of period.                                | Sluggish during first days, short moves or failure to move; daily and longer movements later. | Reduced callow brood emerged at start of period; larval brood passed through principal growth during the period.                           |
| III<br>July 8 to July 25          | Long pouch hanging from vines, around slender tree, 7 M. from ground.                 | Largely minimal; on certain days no raiding at all, on others slow start or beginning in afternoon.     | No colony movement.   | Mature larval brood enclosed at beginning of period, passed through pupation. New brood (eggs, newly hatched larvae) seen on 7/23.         |

a hollow log. (Examination of the bivouac on the two preceding days disclosed that the larval brood was now moderately grown and fairly well distributed through the cluster.) The raid of July 4 toward the east was quite vigorous, with a major swarm division, and in the afternoon there was a new push from the bivouac toward the south; however, no bivouac-change occurred. On July 5 the raid was vigorous, and that evening the colony moved along this route to a new bivouac site in the distance.

In succeeding days the increased status of activity continued even more markedly than before. July 6 found the colony raiding vigorously to the southwest, with a swarm division in mid-morning and a new foray toward the southwest which developed after 3:00 P.M. The colony moved that evening over the latter route, to form an elevated bivouac hanging in a mass of vines about 130 meters from the previous site. When the cluster was broken open the larval brood was found to be far advanced in development, with some of the largest individuals already enclosed. On July 7 there was a vigorous raid southward, and in the evening a movement occurred to a site about 75 meters distant, where a pouch bivouac was formed against the side of a large tree six meters from the ground. On the following day, July 8, the raid was unusually well developed. At the bivouac site cocoon-spinning was the prevalent activity, with columns of larva-carrying workers much in evidence between the bivouac and a bark-strewn area between tree roots. That evening the colony moved only about 40 meters, to form a pouchlike cluster

suspended from a mass of vines seven meters from the ground around a small tree. Since an inspection of the brood during the movement revealed that most of the mature larvae already were enclosed, the beginning of a new statary period appeared to be at hand.

Upon my return to the Island on July 13 from work in another locality, this colony was found as expected, bivouacked in the same place and in nearly the same form in which it had been left on July 8. The records of succeeding days concerning raiding may be briefly summarized as follows: 7/13, a moderately developed raid to the south; 7/14, a moderate raid to the northeast; 7/15, a slowly developed raid to the northwest; 7/16, no raiding; 7/17, a raid to the west, not under way until after 10:30 A.M.; 7/18, a limited raid to the northwest; 7/19, a rather feeble raid to the north, started in the afternoon; 7/20, no raid; 7/21, a relatively small raid toward the north; 7/22, a moderately developed raid to the southeast; 7/23, no raid; 7/24, a moderate raid toward the southeast; 7/25, a moderately developed raid to the south.

When the cluster was opened with a pole on July 23, samples of the brood which fell to the ground were found well advanced in pupation. In addition there were specimens of a new brood, packets containing eggs as well as some newly hatched larvae. On July 25, when my stay at the Island closed, the colony was still bivouacked at the site first occupied on July 8.

During an observation period of 39 days this colony evidently passed through two different statary periods separated by an interval of (modified) nomadic activity. The ten statary days at the outset were regular (i.e., in the usual pattern), with reduced raids in various directions from the site, but without raids on occasional days. Also typical was the presence of an enclosed pupal brood (the appearance of which as callows marked the end of the period) and a new brood of eggs and young larvae. However, on June 23 the major portion of the pupal brood was removed, so that the complement of callows delivered into the colony at the end of the period was only a small part of the number ordinarily appearing.

In the following 12 days the behavior of this colony resembled the pattern already described as nomadic, although at first there were marked differences from the typical course of events. The first raids of the period were relatively small, throughout the first week the bivouac-change movements were shorter than usual and sluggish in their development, and on certain of the days no movement occurred. However in the latter part of the period raiding increased in vigor, and during the last five days bivouac-change became a nightly event. Two facts concerning the brood are notable: in the early part of the period a greatly reduced callow brood was present; and in the latter part of the period a nearly complete larval brood was approaching maturity. The first condition, as suggested above, appears to have been involved in the alteration of initial nomadic events in colony behavior; the latter condition in the appearance of typical nomadic activity during the latter part of the period.

That the relatively brief departure of this colony from the typical *burchelli* pattern depended upon special and temporary conditions is also shown by circumstances in the statary period last observed. The beginning of statary behavior came, as usual, when the largest part of a mature larval brood became enclosed. It may be surmised that upon the eventual appearance of this substantially unreduced brood as callows, the colony was able to enter a fully vigorous nomadic status.

No great importance attaches to the fact that the two statary bivouacs of this colony were established in exposed places rather than within secluded places such as hollow logs or trees as is typical of burchelli (and hamatum) colonies. In the northeastern section of the Island where the colony operated at the time, the forest is largely second growth in which hollow standing trees or logs are few and well scattered. In the southern and western sections where the timber is heavier, hollow trees and logs are relatively frequent in occurrence, and accordingly statary bivouacs established there by burchelli colonies are rarely exposed.

*Evidence for a reduced colony excitement in the statary period:* The difference in burchelli raiding during statary and during nomadic times is, on the whole, very striking. Typically, as already described (Schneirla, 1944a), in the nomadic period raiding begins at dawn, a directionalized swarm is set up relatively early, and the extensive development of the foray is indicated both by the great size of the initial swarm and by the later formation of sub-swarms. In contrast, in the statary period morning activities begin sluggishly, directionalized mass activity usually develops slowly, and the swarms are relatively small and seldom reach the point of even a minor division. A comparable difference has been reported for *E. hamatum* (Schneirla, 1938), in which, because of the nature of raiding, the contrast is more strikingly apparent than in burchelli.

However, one nomad-statory difference stands out more prominently in burchelli than in hamatum; namely, a marked reduction of activity outside the bivouac during the middle of the statary period. In four of our longer burchelli records which cover the first two weeks or more of this period, in each instance raiding occurred on every one of the first six days. Then, as in the first and second statary stops of our 38I colony (Table I) and as in Müller's colony, occasional raidless days began to appear. With only minor variations in different instances, in general it can be said that the interval from the seventh to the sixteenth days (roughly) of the statary period is a time of distinctly lowered colony excitement. Frequently then the Ecitons spread out in the morning around the bivouac itself, but in insufficient numbers to form a directionalized swarm, or a raid may not get under way until afternoon. Thereafter, in the terminal days of the statary period, daily raids appear to be the rule.

Through the described interval of reduced external activity, there are reductions in certain internal conditions of the burchelli colony. When a colony enters the statary period its mature larval brood, although enclosed, has not completed its spinning, and the termination of this activity involves an observable stirring of the spinners within their cases during the first few statary days. Although this limited activity of the brood apparently does not affect the workers nearly as much as does the activity of free larvae, laboratory observations suggest that it is a stimulative factor to be reckoned with. During the first few statary days there is a shifting about of cocoons and a stirring of workers holding cocoons which is not observed later, when the cases containing the quiescent pupal brood are held in place by relatively immobile workers over considerable intervals of time. It appears that the reduction of predatory activities typically observed in the intermediate portion of the statary period is due mainly to the virtual disappearance of special stimulation from the brood once it has completed spinning and has pupated.

In the light of the above suggestion, it is significant that raidless days disappear and the forays increase somewhat in vigor during the last statary week, the time during which slight movements of the mature post-pupae are observed and

when increasing numbers of emerged callows are mixing with workers. That stimulation from the brood becomes a factor of importance is suggested in particular by: an observably greater response of workers to cocoons containing motile individuals than to cocoons containing quiescent brood; a greater activity in experimental groups of workers to which numerous callows have been added than in groups to which equivalent numbers of adult workers are added; and the fact that the first nomadic movement of a colony takes place on the very day the major part of its callows appear.

*Function of the burchelli queen:* For the extensive study of the *Eciton* queen I have concentrated upon *hamatum*, especially because its colonies generally are more accessible to study and their capture less arduous than those of *burchelli*. What evidence is available on the queen of the latter species indicates that her properties are similar to those already described for *hamatum* (1944b).

Numerous observations on the condition of broods at different phases of colony activity, involving many colonies, indicate that the *burchelli* queen must lay a given enormous batch of eggs (30,000 +) within a relatively short time, repeating the process at well separated intervals during the rainy season. Each batch of eggs is laid during a statary period, probably within a time of five or six days near the end of the first third of this period. This is evidenced indirectly by the fact that in all colonies accessible to examination at the time no young have been found in the first six days except an enclosed pupating brood; whereas roughly on the tenth day one begins to find packets of eggs and, later in the period, newly hatched larvae as well. Since under the conditions of this study (i.e., rainy season) the queen of each colony apparently delivers without fail a new batch of eggs early in each statary period, the interval between reproductive episodes must be close to 34 days, i.e., from the early part of one statary period to the corresponding part of the next statary period.

At other times within the statary period excepting the relatively brief physogastric interval, and throughout the nomadic period, the queen evidently remains contracted. *Burchelli* queens observed on several occasions at various times in the nomadic period always have been contracted, and attendant circumstances in each instance indicate that some time has elapsed since the queen was last gravid. For example, a single queen was taken on July 22, 1933, from a *burchelli* colony judged to be nomadic from its highly developed raids and from the fact that daily bivouac changes occurred during the three days preceding and the three days following the capture. This queen was fully contracted at the time of capture. The presence of a huge brood of small larvae, all hatched, served to indicate that the colony had emerged only within recent days from a statary period, a conclusion further supported by the presence of many incompletely pigmented callow workers.

Our findings thus oppose the impression of Bruch (1934) and other writers that the contracted *Eciton* queen is a young individual, still non-functional. The regular spacing of broods which is the rule suggests that a single queen, functional at well separated intervals, is the reproductive agent of each colony. Captures of *burchelli* and other *Eciton* queens favor the same conclusion. In the capture mentioned above, only one queen was found, with two broods distinctly different stages of development. The two colonies of *E. burchelli urichi* and *E. burchelli jeanae* which Weber (1941) examined in Trinidad similarly contained but one queen each, in the contracted condition, as did colonies of seven other *Eciton* s.str. species

from which queens were captured in various parts of tropical America (Bruch, 1934). In this terrestrial *Eciton* subgenus a physogastric queen has been taken in only one instance, from an *E. hamatum* colony just ending the first week of a statary period (Schneirla, 1944). From the fact that in the same study 14 queens, all contracted, were taken from as many colonies of that species at various other times in the cycle, the brevity of the queen's delivery interval may be appreciated in contrast to the length of the intermittent resting interval.

An apparent exception was the *burchelli* colony in Kartabo, British Guiana, from which Wheeler and Emerson (Wheeler, 1921) removed two queens on July 21, 1920. Wheeler judged that both individuals were recently emerged young queens, because of their brilliant coloration as well as that "their ovaries were undeveloped as shown by the relatively small size of the gaster." Although neither of these reasons is very dependable, particularly the latter, the conclusion is made reasonable by the fact that large numbers of enclosed mature male pupae were found in the same colony. If these were both new queens, the possibility remains that the "old queen" of the colony may have escaped unnoticed in the general confusion. Strong and agile, these individuals are difficult to detect and capture unless an anesthetic agent is used. Furthermore, since these captures were made near the end of a rather irregular British Guiana rainy season, the circumstances do not seem comparable to those of our study. In the regular Panama rainy season the broods of *burchelli* colonies are very large, contain worker forms exclusively, and appear at regular intervals as do those *hamatum* (Schneirla, 1944), indicating the functioning of a single queen in each colony.

#### SUMMARY AND DISCUSSION

Our evidence indicates that the theoretical conception of an *Eciton* nomad-statary cycle previously established for *E. hamatum* also holds for *burchelli* despite marked differences in the behavior pattern of the two species.

Certain differences are apparent in the timing of the cycle in these two terrestrial *Eciton* species. In *E. hamatum* we have found (1938) that the phases are nearly equal in length, the nomadic interval about 16 to 18 days and the statary interval about 18 to 20 days. In *burchelli* the nomadic phase appears to be much shorter, approximately 11 to 13 days, and the statary phase considerably longer (21 to 24 days) than in *hamatum*. The clarification of this species difference in the cycle demands further evidence.

Probably more important is the fact that the total length of the behavior cycle appears to be nearly the same in the two investigated species, approximately 35 days from the beginning of one nomadic phase to the beginning of the next one. Since our evidence indicates that the initiation of each new nomadic phase depends upon influences set into effect with the emergence of a new callow brood, this species similarity in the total cycle length would appear to depend upon an equivalence in the spacing and in the developmental period of the brood in the two species.

The existence of various combinations of regularly concurrent events in the life of *burchelli*, noted in part by Müller in his study of a single colony in Brazil, is well established by our results. Significant parallels regularly observed in colony behavior and in the intra-colony situation are the following: One, during approximately one week before a colony breaks out of the statary phase, callows in increasing numbers are removed from their cocoons, and on the day of the first nomadic movement it is noted that the bulk of the mature brood has emerged. Two, when nomadism begins a new brood is present, then at an early stage of larval development. Three, as this brood passes through its larval period the

colony remains nomadic and becomes increasingly active, but four, when the larvae reach a point of sufficient maturity to permit spinning, the colony stops in place and its raiding activity falls off in vigor on the day the greatest part of the brood becomes enclosed. Five, in the statary phase, once the brood has completed its spinning and is pupating the colony falls to its lowest point in raiding activity, and sluggish raiding prevails until pupae have matured sufficiently to begin stirring within their cocoons.

In many *burchelli* colonies studied for periods of two days or longer in the Panama rainy season these parallel events in colony behavior and in brood condition have appeared invariably. Without much question a causal relationship is involved.

How do our findings compare with Müller's? His results fall smoothly into our concept of a cycle of events, when we regard the first three days of his record as the end of a nomadic phase and the ensuing days as statary. However, our interpretations differ, particularly it seems, because Müller's facts were incomplete. At the start he was insufficiently impressed by the concurrence of brood enclosure and cessation of colony movement, hence later when the colony remained immobile despite repeated attempts to stir it up, he was led to explain the inertia in terms of a presumed difficulty of moving a queen. Now as an explanation of Eciton colony movement or its absence the queen-transport hypothesis seems quite barren (Schneirla, 1938; 1944b). In a colony movement, the queen generally appears at a time too late to influence the issue of bivouac-change; moreover, she evidently becomes physogastric *after* the colony has become statary, and returns to the contracted condition long before the colony resumes its nightly marches. Actually, colony mobility seems to depend upon *condition of the brood*, a matter which Müller restricted to variations in raiding.

Müller's hypothesis for differences in raiding was essentially teleological. Having noted a decline in raiding when the brood became enclosed and in pupation, he thought "nothing seems more natural than that the food need of the colony is reduced and the ants go out correspondingly less for booty after the larvae are enclosed" (p. 87). Had the study continued to the point of resumed nomadic activity, the concurrent appearance of hordes of voracious callows might well have appeared to furnish additional support for this view. However, a closer examination of this telic food-need hypothesis shows that it merely calls attention to an existing combination of circumstances (i.e., the greatest raiding and greatest mobility of a colony occurring when the brood is active and is feeding), without explaining how these circumstances happen to arise consistently together. It is quite apparent that the pattern of events is one of great adaptive value; with that granted, we contend that the essential causes of the combination lie in the evolutionary background of the species. There food-scarcity factors may well have been involved; however, we find no indication that they are required to make the adaptive pattern effective in the current situation.

For example, a *burchelli* colony with a maturing larval brood becomes statary (i.e., stops moving; reduces its raiding) on and after the day when this brood has spun its filmy outer envelopes. The total food requirement of the colony must fall considerably with this subtraction of brood feeding, but it is difficult to see how that fact in itself can account for the altered activity pattern of the adult workers. Teleology here tends to obscure and distract attention from the actual causal fac-

tors in the given situation. If it is considered too disingenuous to ask how creatures as psychologically limited as are Eciton workers can estimate colony food needs and act accordingly; at least we may inquire how a food-need hypothesis can account for changes in colony raiding *during* the statary period, when the advanced brood is safely enclosed and the new brood (once it appears as eggs) is not feeding.

The facts require a naturalistic, non-teleological explanation. A theory of this nature which seems to fit the situation has been derived from previous work with *E. hamatum* (Schneirla, 1938). Essentially based upon Wheeler's (1928) *trophallaxis* concept, this theory accounts for changes in the overt behavior of an Eciton colony in terms of periodic changes in the total effect of interindividual stimulation developed within the aggregation. The trophallactic conception takes account of the many ways in which workers may be stimulated tactually and chemically by the brood, in handling or carrying about the young, in licking or feeding them, in passing among the brood or in meeting workers that have been aroused by brood relations such as these, and the like. According to this view, the presence of a brood influences adult behavior to the extent that the young, through their activity and effulgence of chemical stimulation, may increase the activity of workers (with consequent summation effects among the latter), so that activities outside the bivouac are increased correspondingly. Thus the critical factor is the amount of incidental stimulation furnished by a brood, whether or not it is consuming food at the time.

Only on this basis can we account for changes in colony behavior in the absence of brood feeding. In particular, during the statary phase raiding falls to a low point after the first week and remains there until the last week, although brood feeding seems absent throughout. It is probable that these changes in colony behavior depend mainly upon differences in tactual stimulation from the brood. At the beginning of the period the brood drops greatly as an excitatory factor when the larvae are first covered with silk, however a partial stimulative effect is furnished by the spinning activities until the cocoons are finished. Thus daily raids occur although the brood has ceased to feed and the colony does not stand in need of food on its account. These raids are inferior to nomadic raids, since larvae stimulate workers more when they move freely and present an open chemostimulative cuticular surface than when their movements are reduced and only the dry, silky envelope surface is presented. Later in the period when the brood is finished with spinning and is pupating, its members quiescent within their cases become passive contents of the bivouac, then, lacking the brood increment in colony "drive" the workers fall to a new low in their raiding. Colony activity continues at this low level until the brood again becomes a functional source of stimulation.

This interpretation may be extended to explain a resurgence of activity in burchelli colonies about one week before the statary period ends. A considerable portion of the pupal brood is then nearly mature, and capable of slight movements (within the cocoons) which observably stimulate the workers. As laboratory observations show, this stimulative effect is exerted *before* the opening of cocoons has begun. The slight tarsal and antennal twitchings and limited dorsoventral contractions of the enclosed callows are visible to the naked eye, and can affect the workers through pressing upon cocoon walls. Workers in an artificial nest containing motile enclosed brood carry the cocoons about and handle them more, and

display greater activity in general, than do workers provided with an equal number of enclosed immobile pupae. As a control, mature (pigmented) pupae immobilized by needle puncture of the head do not exert this effect.<sup>8</sup>

The irradiation of such excitement through the bivouac heightens the general responsiveness of the colony, so that a greater reactivity to light causes raids to begin more promptly after dawn than was previously the case, and leads to the impressing of larger numbers into each foray. On the other hand, while the brood remains inactive "colony drive" depends upon the interstimulative relationships of adult workers alone, and must remain at a minimum until a new source of augmented stimulation appears.<sup>9</sup>

In *burchelli*, as in *hamatum* (Schneirla, 1938), the appearance of a new callow brood furnishes the impulse for the resumption of nomadic behavior. Then vigorous daily raids occur, each reaching the threshold of bivouac-change movement (Schneirla, 1944a). That this change depends upon the amount of stimulation received from a large callow brood is indicated by our results from a colony deprived of approximately three-fourths of its mature pupal brood late in the statary period. A recognizable nomadism appeared in this colony when the remaining callows emerged; however the daily raids fell below normal standards, and during the first week bivouac-change movements tended to be short in distance or not to occur. Then the colony became more active and its movements attained the normal frequency and vigor. The latter change evidently depended upon a new brood factor.

Although the emergence of a callow brood initiates the nomadic phase of the behavior cycle, this factor apparently does not remain a major source of "drive" for more than a few days. Observations show that callows then behave more like ordinary workers, less excitatory in their effect upon the colony. The above results are what would be expected from a major reduction of the callow-stimulation factor. However, the reinvigoration which occurred in our experimental colony suggests that after about one week of nomadic activity a new excitatory agency becomes effective. Evidently this is introduced through the sufficient growth of a new larval brood whose presence at that time we have found invariable in both *burchelli* and *hamatum* (1938). When a colony first takes to the march the individuals of this brood are very small, gathered together in packets for the most part, and (under laboratory observation) low in stimulative effect upon workers. After a few days when the larvae are larger, this brood is found more widely distributed

<sup>8</sup> In both *burchelli* and *hamatum* (Schneirla, 1934; 1938) the cocoons are opened and the brood emerges as a result of workers' responses to the stirring of mature individuals. Although adults holding quiescent young stand quietly with their burdens, others clutching dynamized young frequently shift their hold and move about restlessly, thereby causing other workers to catch hold of the cocoons. The excitement increases reciprocally, and in the tugging and regaining of lost holds the envelope is soon torn open. In this operation the arousal of increased movement of the callow both augments the struggle of the workers and incidentally facilitates tearing of the case.

<sup>9</sup> Ecitons are very simple creatures psychologically, unable to "realize" needs, but able only to react directly in simple immediate ways to specific, present stimulation. For example, *burchelli* colonies regularly stage their raids in the daytime and not at night when these (almost exclusively chemo-tactical) animals could find their prey readily. This condition prevails regardless of existing food requirements in a colony, simply because the photokinetic effect of light is essential to arouse a foray, and in the absence of light Ecitons become lethargic (Schneirla, 1944a). It should be added that the arrival of daylight is least effective upon a colony when intrinsic social stimulation is at its lowest level in the activity cycle.

through the bivouac, and observably excitatory to the workers. This excitatory effect evidently increases in time as the larvae grow, since in the latter part of the nomadic period raiding tends to increase in vigor and numbers of participants. Because of the influence of the more precocious larvae early in the period, the larval factor must overlap that of the callows to some extent as a stimulative factor promoting nomadism, later fully replacing the callows in this rôle. Finally, as in hamatum, when the bulk of the larval brood becomes enclosed and brood stimulation is greatly reduced, the burchelli colony enters a new statary phase.

It is clear from our findings that burchelli colony movements occur in a regular manner, in a predictable series which begins and ends rather abruptly, rather than occurring haphazardly as many writers have supposed. This fact alone would suggest that their basis is not alimentary, dependent upon the supply of food in given areas, as Heape thought (1931). No essential relationship is found between the food supply in given areas and the mobility or immobility of Eciton colonies which would suggest an alimentary basis for the functioning of the *existing* behavior pattern. Instead, the occurrence of the described nomadic and statary activity phases in alternation seems to depend upon the reproductive condition of the colony, and fundamentally upon the reproductive cycle of the queen (Schneirla, 1944b). Since the basic cause of the existing behavior pattern is an intraorganic, gametic factor which functions cyclically, we may say that Eciton behavior conforms to one of the two important criteria generally accepted as essential to migration.

It will be recalled that Heape and others regard migration as a group movement which is reversible and which has its basis in a reproductive process, reserving the term "emigration" for transferral movements which are not regularly reversible and are based on non-gametic processes. Broadly speaking, the latter term would seem applicable to the unitary Eciton bivouac-change movement, in which a colony changes its dwelling place in a single continuous action. Yet this usage takes certain liberties with Heape's meaning of "emigration," since we have found that even the single bivouac-change movement is clearly dependent upon gametically-based conditions in a colony. If the concept does not fit the unitary Eciton movement, it is far less adequate, indeed very inadequate, to denote the series of movements occurring in a given nomadic phase, in which changes of bivouac take place daily in dependence upon a continuous excitation grounded in reproductive processes. For a given nomad-statory cycle, in which a colony moves from one statary bivouac to the next in a regular and predictable manner, the term "migration" would appear appropriate.

By emphasizing gametic factors we do not exclude the possibility that the evolutionary rise of the Eciton behavior pattern may have involved alimentary factors in an important rôle. For example, if an ancestral form ever existed which failed to move at all or undertook very irregular movements while a larval brood was present, an inevitable shortage of food for the growing members of the colony might well have endangered species survival in favor of variant forms. It cannot be denied that alimentary factors may have exerted a critical influence upon the *phyletic* origin of the Eciton behavior patterns.

However, the question of evolutionary background must not be confused with the problem of accounting for the functioning of the *existing* behavior pattern. In the causation of the existing pattern the food-supply factor plays only a very

secondary rôle, in that the quantity of booty found in the early hours of daily raiding influences the direction of the day's foray; however, *booty haul cannot determine whether or not a bivouac-change movement will occur*. Eciton behavior as it functions in existing species presents many features which are adaptive in rather precise and effective ways: in particular, a colony is mobile when its food requirements are greatest, and at rest when they are least; a colony is at rest when the queen is physogastric, thus incidentally shielding her from external hazards, and on the move when she is contracted and able to travel with greater security. These concurrent conditions presumably arose through a long selective process in evolution, and assuredly have great adaptive value both individually and collectively; but we cannot regard them as factors which play a controlling part in the existing behavior pattern. The mechanism for the latter is a dynamic trophallactic process which depends in particular upon given reproductive properties in the queen, and upon given stimulative and reactive properties in brood and workers. The reproductive factor is of paramount importance for the current appearance of the Eciton pattern.

One of the major criteria of migration is satisfied by the fact that Eciton movements occur regularly and periodically on an intraorganic (i.e., reproductive) basis. The second major criterion employed by Heape and generally accepted is that a directionality exists in one phase of migration which is reversed in another phase. Thus salmon characteristically migrate to sea as young individuals, and return upriver as mature adults; many birds of the northern hemisphere move southward in autumn and northward in spring. At first sight Ecitons appear to lack completely this "horizontal" type of directionality. To an extent however their movements exhibit a horizontal directionalization, in that a mechanism already described insures that a colony will not double in its tracks but must take a new route in each successive daily movement. Still this control over direction is a limited one, which cannot prevent changes in the principal course of a given succession of nomadic movements. Moreover, there exists no indication of a reversal of the direction of horizontal progress from one phase of the cycle to the next.

At this point it may be asked what underlies the occurrence of directionality and its reversal in the generally accepted instances of migration. The basis is not very clear in the current evidence, yet perhaps we may say that one direction of progress in a migration is an expression of given responses to external conditions, and that in the opposite phase of the cycle these responses are changed or reversed, in response to changed or reversed external conditions. The different responses to external conditions in the two phases of the cycle may be regarded as dependent upon different (perhaps reversed) intraorganic conditions.

This interpretation suggests to us one characteristic of the Eciton pattern which may be regarded as the biological equivalent of a direction reversal in the usual sense. We have noticed that during a nomadic phase *burchelli* (and *hamatum*) colonies tend to bivouac next the surface of the ground, that toward the end of this phase the colonies tend to cluster at a distance from the ground, and that stately bivouacs almost invariably are formed in elevated positions. This difference, together with the fact that nomadic bivouacs generally are exposed whereas stately bivouacs tend to be secluded, suggests that colonies of terrestrial Eciton species respond differently to environmental conditions according to the given phase of their activity cycle. The outcome may be considered an instance

of "altitudinal" migration, somewhat comparable to animals (e.g., some birds) which migrate from valley to hill and return in a different phase of their cycle without any particular reversal of the latitudinal direction taken in the ascent. Apparently the limited ascent and descent during different stages of migration in terrestrial Ecitons somehow has its basis in changed intra-colony conditions centering around the brood. At present both the nature of this basis and of the external conditions involved in the altitudinal movement are matters of conjecture.

The writer believes that a general theory of animal migration cannot be worked out without careful attention to patterns which conform only partially to the traditional vertebrate examples. Thus Williams (1943) and Fraenkel (1932) have presented convincing reasons for including in the general evidence the movements of certain insects such as many lepidopteran and orthopteran species. The Ecitons, it would appear, must also be included. These insects seem to represent an example of primitive or rudimentary migration in which the basis of the pattern is present without a highly specialized superstructure like that found in animals such as many birds, permitting a directionalization of horizontal locomotion. On this view, the organic "drive" basis of migration is fundamental, but may exist in the partial or complete absence of specialized factors permitting a directionalized and reversible movement.<sup>10</sup>

It should be emphasized that the army ants are exceptional among migrating animals in that their movements are carried out by individuals which are not themselves responsible for the origination of the basic drive factor. Indeed the actual process of locomotion is brought about in the workers in a very indirect manner, which involves not only an incidental stimulative function of the brood as intermediate process, but also effects itself through a worker activity (raiding) which is qualitatively different from the true colony movement. As we have seen (Schneirla, 1944a), the very different patterns of swarm-raiding and column-raiding may both lead into migration provided that other factors are involved which are basically independent of the workers themselves. Yet these other factors, the fundamental contribution of the queen and the intermediate rôle of the brood, are smoothly interrelated with the "effector" function of the workers in a highly stereotyped manner, so long as the optimal environment furnished by regular rainy-season conditions exists. What differences may appear in the pattern under the non-optimal conditions of dry-season weather, or in an environment of irregular annual rains, is a problem for further investigation.

#### CONCLUSIONS

1. The cyclical pattern of nomadic and statary behavior described for the swarm-raiding species *E. burchelli*, and previously reported for the column-raiding *E.*

<sup>10</sup> Woodbury (1941), in advancing his *periodic-response theory* for migration in general, points out that the visceral rhythms which are held basic to migration may be correlated with environmental periodicities in a variety of ways and to different extents in different animals. In the Ecitons, although seasonal differences in migratory activities apparently exist, and although the day-night periodicity is involved in the arousal of separate movements, evidence is lacking that the timing of the nomad-statory cycle itself is dependent upon extraorganic periodicities. However, for the queen's reproductive cycle which the present writer (1944) has found basic, Weber (1943) suggests that the lunar cycle may play a surrogate rôle. A number of circumstances oppose this possibility in the investigated species, in particular the fact that at a given time colonies may vary considerably in the phases of their brood and activity cycles, also the fact that the duration of the queen's ovulation cycle falls close to 35 days rather than approximating 28 days as the lunar-cycle hypothesis seems to demand.

*hamatum*, may be considered typical of terrestrial Eciton species. Species differences exist in the relative length of the phases of this activity cycle.

2. The basis of the pattern in *burchelli*, as in *hamatum*, is in the rhythmic reproductive function of the colony queen. This factor expresses itself through periodic changes in the stimulative (i.e., trophallactic) effect of broods upon the workers, and thus indirectly controls the presence or absence of regular daily colony movements.

3. Since these Eciton movements occur in a regular and periodic manner, on an intraorganic (reproductive) basis, they may be considered a "primitive" or rudimentary instance of migration. In the army ants there exists a biological equivalent of directionalization of migration and its reversal, in the form of an altitudinal shifting of the home site in different phases of the activity cycle.

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# THE RESISTANCE AND ACCLIMATIZATION OF MARINE FISHES TO TEMPERATURE CHANGES. II. EXPERIMENTS WITH FUNDULUS AND ATHERINOPS<sup>1</sup>

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## INTRODUCTION

In an earlier paper (Doudoroff, 1942) a detailed study of the resistance and acclimatization of the marine fish *Girella nigricans* to extreme high and low temperatures was presented. Some parallel experiments on the temperature tolerance of the relatively hardy species *Fundulus parvipinnis* and of the very delicate *Atherinops affinis* are reported here. While no attempt has been made to investigate fully the causes of death on chilling, some observations are recorded which bear on this problem.

The literature on the resistance and acclimatization of fishes and of other animals to low and high temperatures has been reviewed in the earlier publication. Of papers which have come more recently to the author's attention, those of Brett (1941, 1944) and of Fry, Brett and Clawson (1942) on the lethal limits of temperature for various fresh-water fishes must be mentioned. The importance of acclimatization as a factor to be considered in delimiting the temperature tolerance of these fishes was demonstrated fully. Certain early assumptions relative to the mortality rate at lethal temperatures (namely, that death occurs almost always within 12 or 14 hours even at low temperatures) and relative to the rate of acclimatization to low temperatures were not fully substantiated and are not entirely in accord with the author's observations on marine fishes.

The survival of some hardy fishes at very low, sub-zero temperatures in a state of seemingly suspended animation formerly had received more attention than death at low temperatures well above 0° C., and frequently has been termed "anabiosis" (Borodin, 1934; Schmidt, Platonov and Person, 1936). This term is misleading. The seemingly lifeless condition of supercooled and superficially frozen fish may be due to reversible injury to the central nervous system. The sensitive automatic mechanism of the heart also may be affected (Battle, 1926). There is no evidence that tissue respiration and all other vital functions are arrested (see also Kalabuchow, 1935), nor that the so-called anabiotic state can persist for a very long time. Therefore, this state may not differ fundamentally from chill-coma at above-zero temperatures and even may be closely related to heat-coma. The low metabolic rate at the lower temperatures and the hardness of the species in which "anabiosis" has been observed account for the fact that the coma, with attendant respiratory and possibly circulatory disturbances, can be more prolonged at sub-zero temperatures.

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Weigmann (1936) has contended that the death of fish which he super-cooled to very low temperatures was caused by respiratory disturbances. These he believed to be due to a direct effect of cooling upon the respiratory center of the brain, although other possibilities also were suggested. The observation that the animals could be revived, after return to normal temperatures, by the administration of artificial respiration was advanced as one of several arguments in support of the opinion that some respiratory disturbance was the cause of death. It will be shown that other factors can play an important role in the causation of death by chilling.

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#### METHODS AND MATERIAL

The methods and equipment used in the evaluation of temperature tolerance were those employed in the experiments with *Girella* (Doudoroff, 1942). Briefly, the measures of relative tolerance adopted are the lower and upper median tolerance limits ( $T_m$ ), defined as the low and high temperatures, respectively, which are lethal to just 50 per cent of the experimental animals in a given specified period of exposure. These values are estimated, when necessary, by interpolation, being then based on observed percentages of specimens surviving at two successive, constant test temperatures, usually 1° C. apart, one of which is lethal to more than half, and the other to less than half of the animals tested. Estimation of median tolerance limits for a series of time intervals permits verification of the individual values and frequently evaluation of the ultimate median tolerance limit, that is, the temperature which is tolerated indefinitely by 50 per cent of the animals.

Usually the fish were acclimatized and tested in constant-temperature aquaria supplied with running sea water, but in experiments in which the salinity of the medium was varied, jars with standing, continuously aerated water were used. Unless otherwise denoted here, the medium was sea water.

*Fundulus parvipinnis* Girard inhabits shallow and enclosed bays, sloughs and estuaries. Adults of both sexes, taken in Mission Bay near San Diego, were used. Average and minimal temperatures in the habitat of the species in this locality probably do not differ greatly from those in open water at the Scripps Institution of Oceanography nearby (Doudoroff, 1942). Maximal temperatures to which *Fundulus* may be exposed for brief periods in summer are considerably higher, however, than those recorded in open water, temperatures of 30° C. or higher probably being not uncommon during low tide in some places which are frequented by these active fishes.

Young *Atherinops affinis* (Ayres) were taken near the Scripps Institution at La Jolla, where, like *Girella*, they are common in open water near the shore. This species also is common in bays and estuaries, but, unlike *Fundulus*, it apparently does not frequent very shallow inlets and sloughs of the coastal marshes, where temperatures much higher than those in open water occur frequently. Thus, *Atherinops* probably lives under approximately the same temperature conditions as *Girella* in the same general locality. According to Jordan, Evermann and Clark (1930), the range of distribution of *Atherinops* extends along the "coast of California and

north to Oregon," *i.e.*, further north than that of *Girella*, while that of *Fundulus* extends "from Point Conception" (actually it extends somewhat further north) "southward to Lower California."

Comparative tests showed clearly that *Atherinops* is less resistant and *Fundulus* is more resistant than *Girella* both to reduced salinity (dilution) of the medium and to asphyxiation (in tightly stoppered bottles in which the three forms were confined together). In each of these tests, which were performed with groups of specimens of equal average weight, all specimens of each of the less resistant species succumbed long before any of the specimens of the next more resistant species.

Only a relatively small number of the delicate and active *Atherinops* could be brought to the laboratory and kept for long periods in good health. *Fundulus*, although hardy, is subject to disastrous epidemic diseases in the laboratory (Wells and ZoBell, 1934) and often is heavily parasitized. Care was taken to select for experimentation only healthy specimens without parasitic isopods on the gills.

## RESULTS

### *Effects of extreme temperatures*

*Fundulus* and *Atherinops* were affected by exposure to extreme high and low temperatures much as were *Girella* (Doudoroff, 1942), but the following important differences were noted.

If returned to a normal temperature within a few minutes after the onset of heat-coma, *Fundulus*, unlike *Girella* and *Atherinops*, often showed partial, temporary recovery, and sometimes complete recovery.<sup>3</sup> The administration of artificial respiration, by passing a stream of water through the mouth and over the gills, favored the recovery.

At slowly lethal temperatures primary chill-coma, that is, temporary cessation of all movements almost immediately following transfer to a low temperature, often was more prolonged in *Fundulus* than in *Girella*. *Fundulus* sometimes showed spontaneous, temporary recovery after remaining in a state of complete primary coma for more than one hour.<sup>4</sup> Specimens which failed to resume respiratory movements within two hours after cooling never moved thereafter at the low temperature. If returned to a normal temperature, these fishes were able to recover after longer periods, but recovery after three hours was observed only in one instance. However, recovery was obtained by the administration of artificial respiration together with warming when warming alone was not sufficient. This is illustrated by the following experimental results. Fourteen specimens, which had been held at 20° C., were exposed to 2° C., at which temperature none recovered from primary coma. After three hours the fishes were returned to 20° C., and artificial respiration was administered to one half of the specimens. All of the latter specimens resumed respiratory movements, and all but one recovered completely and were alive after three days. The seven specimens to which artificial respiration was not administered showed no recovery whatsoever.

<sup>3</sup> Recovery has been obtained with other hardy fishes, *e.g.*, *Gillichthys mirabilis* (Sumner and Doudoroff, 1938). Injury to the integument and paralysis of body muscles sometimes were noted in *Fundulus* which had recovered partially after exposure to very extreme high temperatures. At these temperatures bleeding of the gills was observed occasionally.

<sup>4</sup> Such temporary recovery after prolonged primary chill-coma was observed in tests with other hardy species, *e.g.*, *Leptocottus armatus* Girard.

If spontaneous recovery from primary chill-coma occurred at the low temperature at which the coma was produced, *Fundulus* always survived at this temperature for more than 12 hours before the onset of permanent, secondary chill-coma. For example, of 24 specimens which were transferred from 20° to 3° in a preliminary test only 14 recovered from primary coma, yet all of these survived for 12 hours, and 9 were still alive after 24 hours. The surviving specimens righted themselves and appeared normal. Later, however, they became seemingly stiff and emaciated, their eyes becoming deeply sunken. This appearance suggested osmotic dehydration of the tissues. Secondary chill-coma followed. If warmed thereafter, *Fundulus* sometimes showed brief activity, but complete recovery was unusual.

In *Atherinops* temporary recovery after complete primary chill-coma was observed rarely. Violent paroxysms, loss of equilibrium and partial inactivation often occurred after cooling and could be followed by recovery, but if movements ceased entirely for more than a few minutes, the coma was always permanent, and after one hour this species never recovered even on transfer to a normal temperature.

Permanent cessation of respiratory and other movements, either spontaneous or induced by mechanical stimulation, was regarded as the onset of death or end-point of survival, as in the experiments with *Girella*. With *Atherinops*, as with *Girella*, determinations could be made of lower median tolerance limits for exposure periods of one hour or more. Inasmuch as *Fundulus* sometimes recovered from primary chill-coma after periods longer than one hour, the shortest exposure period adopted for cold-tolerance tests with this species was three hours.

#### *Time-temperature relation of cold-tolerance in Fundulus*

*Fundulus* collected in late summer and stocked at current sea water temperatures of 19° to 21° were soon transferred to constant-temperature tanks at 14°, 20° and 28°. Many of the specimens held at 14° and most of those at 28° died of disease. The relatively healthy 20°-conditioned fish were used in cold-tolerance tests after a sojourn of 30 days or longer in the constant-temperature tank. The experimental results are presented in Table I. Since all specimens which survived for 3 hours survived also for 12 hours, the data for these exposure periods appear in a single column.

TABLE I

*Per cent survival of 20°-conditioned Fundulus at various low temperatures and the estimated lower median tolerance limits ( $T_m$ ) in relation to time (duration) of exposure to the test temperatures*

| Date 1940 | No. of fish and ratio ♂: ♀ | Mean length (cm.) | Test temp. (° C.) | Per cent surviving after |       |        |        |        |        |        |        |
|-----------|----------------------------|-------------------|-------------------|--------------------------|-------|--------|--------|--------|--------|--------|--------|
|           |                            |                   |                   | 3 to 12 hours            | 1 day | 2 days | 3 days | 4 days | 5 days | 6 days | 7 days |
| 8/28      | 12 (9:3)                   | 6.8               | 7°                | 100%                     | 100%  | 100%   | 100%   | 100%   | 100%   | 100%   | 100%   |
| 9/4       | 14 (10:4)                  | 6.7               | 6°                | 100                      | 100   | 100    | 100    | 100    | 93     | 86     | 86     |
| 9/11      | 12 (9:3)                   | 6.7               | 5°                | 100                      | 100   | 100    | 92     | 67     | 25     | 0      | 0      |
| 9/22      | 12 (8:4)                   | 6.7               | 4°                | 100                      | 100   | 100    | 83     | 25     | 0      | 0      | 0      |
| 9/18      | 16 (12:4)                  | 6.7               | 3°                | 87                       | 81    | 50     | 0      | 0      | 0      | 0      | 0      |
| 9/27      | 14 (10:4)                  | 6.7               | 2°                | 0                        | 0     | 0      | 0      | 0      | 0      | 0      | 0      |
|           |                            | $T_m$ :           |                   | 2.6°                     | 2.6°  | 3.0°   | 3.6°   | 4.6°   | 5.4°   | 5.6°   | 5.6°   |

The lower median tolerance limits are plotted against exposure time in Figure 1. The sigmoid time-temperature curve of cold-tolerance shown differs strikingly from curves obtained with *Girella* (Doudoroff, 1942, Fig. 1), which showed most of the rise of  $T_m$  with increasing time of exposure occurring during the first day or two. The ultimate lower median tolerance limit for the *Fundulus* apparently is close to  $5.6^\circ$ .

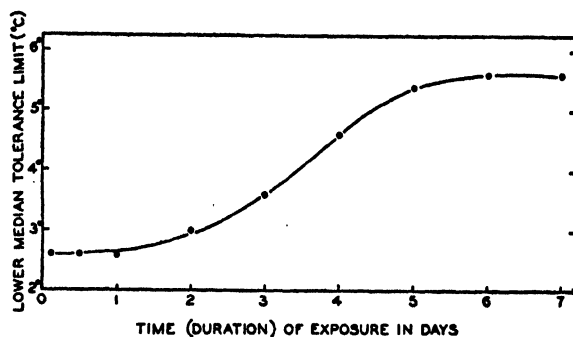


FIGURE 1. Time-temperature curve of cold-tolerance of  $20^\circ$ -conditioned *Fundulus*: Lower median tolerance limits in relation to the time (duration) of exposure to the test temperatures in sea water. Data from Table I.

Comparison of the data with the results of some earlier preliminary experiments and of later tests revealed a small progressive increase of the resistance of the  $20^\circ$ -conditioned *Fundulus* to cold, and especially to the less extreme low temperatures. The cause has not been established, but the change may have been seasonal or related to the sexual cycle.

*Changes of water content at lethal low temperatures and the influence of dilution of the medium and acclimatization on cold-resistance in Fundulus*

The loss of tissue water through osmosis by *Fundulus* which were dying in sea water at slowly lethal low temperatures was inferred from the appearance of the animals. Data presented in Table II, which shows the relative water content (*i.e.*, weight lost on desiccation to constant weight at  $100^\circ$  per unit of the dry weight) for groups of specimens differing in their history, support this inference. The experimental animals which had been exposed to low test temperatures were removed for weighing when they were seemingly near death, but still respiring and responding to stimulation. The results obtained with  $14^\circ$ -conditioned specimens indicate an average loss of more than 23 per cent of the normal water content at the lethal low temperatures ( $1.0^\circ$ – $1.6^\circ$ ) in sea water, but no loss in diluted (45 per cent) sea water. The specimens tested in the diluted water did not appear dehydrated and were still quite lively when they were removed, although they had been exposed to the low temperature ( $1.6^\circ$ ) about twice as long as the specimens tested in sea water. The relatively large  $20^\circ$ -conditioned specimens apparently lost, on the average, 14 per cent of their initial water content at the lethal test temperature ( $3.0^\circ$ ) in sea water.

Osmotic work must be done constantly by marine fishes in their normal medium. Dehydration can result from disturbances of the osmoregulative functions, but

TABLE II

Water content of *Fundulus* after exposure to slowly lethal low temperatures in sea water (SW) and in diluted sea water (45% SW), and that of control specimens taken directly from sea water at the initial acclimatization temperatures. All of the experimental specimens were still alive when removed from the test aquaria. The water content is expressed as units of weight of water per unit of dry weight. The mean values indicated for fish with similar history are weighted according to the number of specimens.

| Acclim. temp. (° C.) | Test temp. (° C.) | Days at test temp. | Test medium | Number and sex of fish | Wet weight (grams) | Dry weight (grams) | Wet wt. - Dry wt. |
|----------------------|-------------------|--------------------|-------------|------------------------|--------------------|--------------------|-------------------|
|                      |                   |                    |             |                        |                    |                    | Dry wt.           |
| 14°                  | 1.0°              | 2                  | SW          | 1 ♂                    | 3.64               | 1.03               | 2.53              |
| 14°                  | 1.6°              | 2                  | SW          | 1 ♂                    | 5.06               | 1.48               | 2.42              |
| 14°                  | 1.6°              | 2                  | SW          | 1 ♂                    | 4.51               | 1.38               | 2.27              |
| 14°                  | 1.6°              | 2                  | SW          | 2 ♂                    | 7.55               | 2.25               | 2.36              |
| Weighted mean:       |                   |                    |             |                        |                    |                    | 2.39              |
| 14°                  | 1.6°              | 3½                 | 45% SW      | 2 ♂                    | 9.84               | 2.45               | 3.02              |
| 14°                  | 1.6°              | 4                  | 45% SW      | 5 ♂                    | 19.29              | 4.57               | 3.22              |
| Weighted mean:       |                   |                    |             |                        |                    |                    | 3.16              |
| 14°                  |                   | Control            |             | 1 ♂                    | 3.51               | 0.86               | 3.08              |
| 14°                  |                   | Control            |             | 1 ♂                    | 3.93               | 0.95               | 3.14              |
| 14°                  |                   | Control            |             | 1 ♂                    | 4.06               | 0.97               | 3.19              |
| 14°                  |                   | Control            |             | 2 ♂                    | 9.91               | 2.43               | 3.08              |
| Weighted mean:       |                   |                    |             |                        |                    |                    | 3.11              |
| 20°                  | 3.0°              | 1½                 | SW          | 1 ♀                    | 6.46               | 1.75               | 2.69              |
| 20°                  | 3.0°              | 2½                 | SW          | 1 ♂, 1 ♀               | 13.63              | 4.02               | 2.39              |
| 20°                  | 3.0°              | 3                  | SW          | 2 ♂                    | 14.65              | 4.32               | 2.39              |
| Weighted mean:       |                   |                    |             |                        |                    |                    | 2.45              |
| 20°                  |                   | Control            |             | 1 ♂                    | 8.62               | 2.13               | 3.05              |
| 20°                  |                   | Control            |             | 1 ♂, 1 ♀               | 15.61              | 4.08               | 2.83              |
| 20°                  |                   | Control            |             | 1 ♂, 1 ♀               | 16.04              | 4.25               | 2.77              |
| 20°                  |                   | Control            |             | 1 ♂, 1 ♀               | 11.60              | 3.01               | 2.85              |
| Weighted mean:       |                   |                    |             |                        |                    |                    | 2.85              |

obviously can be prevented by sufficient dilution of the medium. *Fundulus parvipinnis* probably occurs only in salt water, but it can live in fresh water. The normal osmotic pressure of the blood of this species was not known. From available cryoscopic data for other marine and euryhaline fishes (Dakin, 1912, 1935) it was concluded, however, that 45 per cent sea water probably is roughly isosmotic with the blood of *Fundulus*, or only slightly hypertonic. The results of comparable tests of the cold-resistance of specimens acclimatized to 20° and to 14° and tested in sea water and in 45 per cent sea water (prepared by dilution with water distilled in glass) are presented in Table III. The specimens tested in the diluted water were held in this medium for one day before the test.



At the rapidly lethal temperature of 2° there was no significant difference between the mortality rates of 20°-conditioned specimens in the two media, five of the eight fish in each group having failed to recover from primary chill-coma. At the less extreme low temperatures of 3° and 4°, on the other hand, all the specimens succumbed in sea water before any succumbed in the diluted water. If the mid-point of the 24-hour interval during which each specimen succumbed is taken as the time of its death, the mean survival times at the latter two temperatures are found to be 3.4 days and 4.75 days, respectively, in sea water, while in the diluted water they are 7.0 days and 8.4 days, respectively. The difference between the means for the two media at each of these slowly lethal temperatures is certainly significant, the probability being  $0.9999 +$ .<sup>5</sup> Accordingly,  $T_m$  values for moderately long exposure periods only were clearly influenced by the salinity of the water. The results obtained with the 14°-conditioned specimens, which had been held at 14° for 83 to 91 days, likewise indicate clearly an influence of water salinity upon the mortality rate at a slowly lethal low temperature (1.6°), and also an influence of acclimatization upon cold-tolerance.<sup>6</sup>

*Resistance and acclimatization of Fundulus to heat*

Fundulus used in the heat-tolerance experiments were acclimatized in the three constant-temperature tanks (at 20° for 30 to 51 days, at 28° for 57 to 63 days, and

TABLE IV

*Per cent survival of Fundulus at various high temperatures and the estimated upper median tolerance limits ( $T_m$ ) in relation to temperature of previous acclimatization and to time (duration) of exposure to the test temperatures*

| Date 1940 | Acclim. temp. (° C.) | No. of fish and ratio ♂: ♀ | Mean length (cm) | Test temp. (° C.) | Per cent surviving after |       |        |        |         |         |         |         |         |          |          |  |
|-----------|----------------------|----------------------------|------------------|-------------------|--------------------------|-------|--------|--------|---------|---------|---------|---------|---------|----------|----------|--|
|           |                      |                            |                  |                   | 0.5 hr.                  | 1 hr. | 3 hrs. | 6 hrs. | 12 hrs. | 24 hrs. | 48 hrs. | 72 hrs. | 96 hrs. | 120 hrs. | 144 hrs. |  |
| 9/30      | 28°                  | 12 (5:7)                   | 6.8              | 40°               | 33%                      | 0%    | 0%     | 0%     | 0%      | 0%      | 0%      | 0%      | 0%      | 0%       | 0%       |  |
| 9/30      | 28°                  | 13 (5:8)                   | 6.8              | 39°               | 100                      | 69    | 0      | 0      | 0       | 0       | 0       | 0       | 0       | 0        | 0        |  |
| 9/27      | 28°                  | 13 (5:8)                   | 6.7              | 38°               | 100                      | 100   | 100    | 31     | 0       | 0       | 0       | 0       | 0       | 0        | 0        |  |
| 9/25      | 28°                  | 12 (5:7)                   | 6.7              | 37°               | 100                      | 100   | 100    | 100    | 100     | 42      | 0       | 0       | 0       | 0        | 0        |  |
| 10/1      | 28°                  | 13 (5:8)                   | 6.8              | 36°               | 100                      | 100   | 100    | 100    | 100     | 100     | 100     | 100     | 100     | 100      | 92       |  |
|           |                      |                            | T <sub>m</sub> : |                   | 39.7°                    | 39.3° | 38.5°  | 37.7°  | 37.5°   | 36.9°   | 36.5°   | 36.5°   | 36.5°   | 36.5°    | 36.5°    |  |
| 9/18      | 20°                  | 12 (8:4)                   | 6.7              | 37°               | 0%                       | 0%    | 0%     | 0%     | 0%      | 0%      | 0%      | 0%      | 0%      | 0%       | 0%       |  |
| 9/14      | 20°                  | 13 (9:4)                   | 6.7              | 36°               | 54                       | 54    | 38     | 38     | 23      | 8       | 0       | 0       | 0       | 0        | 0        |  |
| 9/4       | 20°                  | 13 (9:4)                   | 6.6              | 35°               | 100                      | 100   | 100    | 100    | 100     | 85      | 54      | 38      | 31      | 31       | 31       |  |
| 8/28      | 20°                  | 13 (9:4)                   | 6.6              | 34°               | 100                      | 100   | 100    | 100    | 100     | 100     | 100     | 100     | 92      | 85       | 62       |  |
|           |                      |                            | T <sub>m</sub> : |                   | 36.1°                    | 36.1° | 35.8°  | 35.8°  | 35.7°   | 35.5°   | 35.4°   | 35.1°   | 34.8°   | 34.6°    | 34.4°    |  |
| 10/10     | 14°                  | 12 (6:6)                   | 6.7              | 34°               | 42%                      | 25%   | 0%     | 0%     | 0%      | 0%      | 0%      | 0%      | 0%      | 0%       | 0%       |  |
| 10/11     | 14°                  | 12 (6:6)                   | 6.7              | 33°               | 100                      | 100   | 33     | 8      | 8       | 8       | 8       | 8       | 8       | 8        | 8        |  |
| 10/22     | 14°                  | 13 (7:6)                   | 6.7              | 32°               | 100                      | 100   | 92     | 77     | 77      | 69      | 69      | 69      | 69      | 69       | 69       |  |
|           |                      |                            | T <sub>m</sub> : |                   | 33.9°                    | 33.7° | 32.7°  | 32.4°  | 32.4°   | 32.3°   | 32.3°   | 32.3°   | 32.3°   | 32.3°    | 32.3°    |  |

<sup>5</sup> For the method of computing probabilities see Tippet (1931).

<sup>6</sup> Fundulus which had been acclimatized to 28° and then held for 7 days at 36° died within 24 hours after subsequent cooling to 15°. Similar observations have been recorded by Wells (1935).

at 14° for 58 to 70 days) together with those used in the cold-tolerance tests. The experimental data are presented in Table IV.

The time-temperature relationship of heat-tolerance of 28°-conditioned *Fundulus* is somewhat similar to that observed with 20°-conditioned *Girella*, the values of  $T_m$  for exposure periods of 0.5 to 48 hours tending to fall along a straight line when plotted against the logarithm of the exposure time (Fig. 2). The 48-hour

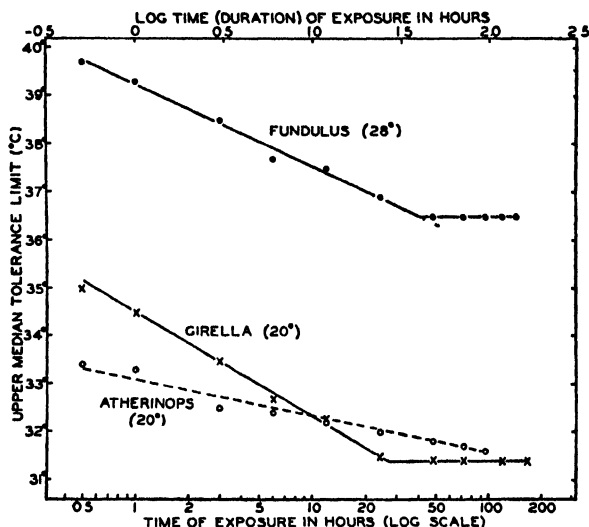


FIGURE 2. Time-temperature curves of heat-tolerance of 28°-conditioned *Fundulus*, 20°-conditioned *Atherinops* and 20°-conditioned *Girella*: Upper median tolerance limits in relation to the logarithm of the time (duration) of exposure to the test temperatures. Data from Tables IV and V and from Doudoroff (1942), Table II.

$T_m$ , or 36.5°, apparently is also the ultimate median tolerance limit. The relationship obtained with 14°-conditioned *Fundulus* is not logarithmic and is not unlike that obtained with 12°-conditioned *Girella*. All deaths occurred during the first day of exposure,  $T_m$  remaining thereafter at 32.3°. The nearly straight-line relationship between  $T_m$  and duration of exposure obtained with *Fundulus* from the intermediate temperature of 20° is quite unlike any others which have been studied. No explanation can be suggested. Nevertheless, the influence of acclimatization upon resistance to heat is shown clearly by the data, and it is evident that *Fundulus*, unlike *Girella*, does not acquire nearly maximal heat-tolerance when it is acclimatized to 20°.

#### *Resistance and acclimatization of Atherinops to heat and cold*

*Atherinops* taken in October, when the surface temperature in their habitat had been close to 20° for some time, were held in the laboratory for three weeks at current sea water temperatures of 19° to 20°, and then in a constant-temperature tank at 20° for one week before tests were begun. The time-temperature relations of tolerance to heat and to cold were studied in the usual manner, but intervals of only 0.5° between successive test temperatures were used in the evaluation of heat-tolerance to ensure desired accuracy. The results are presented in Table V.

Great sensitiveness of this species to cooling is evident. While no simple time-

TABLE V

*Per cent survival of 20°-conditioned Atherinops at various high and low temperatures and the estimated upper and lower median tolerance limits ( $T_m$ ) in relation to time (duration) of exposure to the test temperatures*

| Date 1940 | No. of fish | Mean length (cm.) | Test temp. (° C.) | Per cent surviving after |       |        |        |         |         |         |         |         |
|-----------|-------------|-------------------|-------------------|--------------------------|-------|--------|--------|---------|---------|---------|---------|---------|
|           |             |                   |                   | 0.5 hr.                  | 1 hr. | 3 hrs. | 6 hrs. | 12 hrs. | 24 hrs. | 48 hrs. | 72 hrs. | 96 hrs. |
| 11/6      | 12          | 6.1               | 33.5°             | 42%                      | 17%   | 0%     | 0%     | 0%      | 0%      | 0%      | 0%      | 0%      |
| 11/5      | 14          | 6.1               | 33.0°             | 86                       | 86    | 0      | 0      | 0       | 0       | 0       | 0       | 0       |
| 11/7      | 12          | 6.2               | 32.5°             | 100                      | 100   | 50     | 33     | 0       | 0       | 0       | 0       | 0       |
| 11/2      | 12          | 6.2               | 32.0°             | 100                      | 100   | 100    | 100    | 92      | 50      | 17      | 0       | 0       |
| 11/8      | 14          | 6.1               | 31.5°             | 100                      | 100   | 100    | 100    | 100     | 100     | 86      | 71      | 57      |
|           |             | $T_m$ (upper):    |                   | 33.4°                    | 33.3° | 32.5°  | 32.4°  | 32.2°   | 32.0°   | 31.8°   | 31.7°   | 31.6°   |
| 11/28     | 18          | 6.2               | 11.0°             |                          | 100%  | 100%   | 100%   | 100%    | 100%    | 100%    | 100%    | 100%    |
| 11/20     | 18          | 6.0               | 10.0°             |                          | 100   | 100    | 100    | 94      | 94      | 44      | 22      | 22      |
| 11/16     | 16          | 6.1               | 9.0°              |                          | 100   | 100    | 100    | 94      | 75      | 37      | 6       | 0       |
| 11/15     | 16          | 6.0               | 8.0°              |                          | 100   | 75     | 25     | 0       | 0       | 0       | 0       | 0       |
| 11/14     | 16          | 6.1               | 7.0°              |                          | 87    | 62     | 0      | 0       | 0       | 0       | 0       | 0       |
| 11/17     | 18          | 6.1               | 6.0°              |                          | 56    | 28     | 0      | 0       | 0       | 0       | 0       | 0       |
| 11/16     | 16          | 6.2               | 5.0°              |                          | 0     | 0      | 0      | 0       | 0       | 0       | 0       | 0       |
|           |             | $T_m$ (lower):    |                   |                          | 5.9°  | 6.6°   | 8.3°   | 8.5°    | 8.7°    | 10.1°   | 10.4°   | 10.4°   |

temperature curve of cold-tolerance fits the data closely, the lower median tolerance limit rises progressively with increasing time of exposure for three days, whereupon an apparently constant value of 10.4° is reached. The upper median tolerance limits are plotted against the logarithm of the time of exposure in Figure 2, together with corresponding data for 20°-conditioned *Girella* and 28°-conditioned *Fundulus*.

Some *Atherinops* were held through the winter at current sea water temperatures, which were below 18°, and which averaged 16° during the last month. After subsequent acclimatization for about three weeks to a constant temperature of 18.5° these specimens were found to have lower and upper 24-hour median tolerance limits of 8.8° and 30.5°, respectively. While all the fish died within 24 hours after direct transfer to 31° in this experiment, after a two day sojourn at 30° all survived for 48 hours at 31°, and the majority survived at 32°, thus showing rapid acclimatization to heat. Rapid acclimatization to cold did not occur, but in another experiment with specimens which had been held at 20° and then acclimatized for more than two months to 14.5° and 25.5° a pronounced influence of long acclimatization upon cold-tolerance was demonstrated, the 24-hour lower median tolerance limits for the two lots being 7.6° and 13.5°, respectively.

#### DISCUSSION

Because of the nature and variability of the time-temperature relations of tolerance, prolonged tests often may be necessary in ecological studies of the resistance of fishes to cold and heat. The lower median tolerance limit for *Fundulus*, unlike

those for *Girella* and *Atherinops*, remained nearly constant during the first 48 hours of exposure (6 days in diluted sea water) and rose rapidly thereafter (Fig. 1). Limits of tolerance for uniform exposure periods even longer than 48 hours evidently may not be truly comparable and ecologically significant. Had extended observations not been made, the prolonged temporary recovery of *Fundulus* from primary chill-coma at slowly lethal low temperatures could have been mistaken for permanent recovery.

The time-temperature curves of heat-tolerance for 20°-conditioned *Girella*, 28°-conditioned *Fundulus* and 20°-conditioned *Atherinops* (Fig. 2) are somewhat similar, but they differ greatly with respect to their slope, the temperature coefficients,  $Q_{10}$ , of the velocity of heat injury in these fishes being about  $4 \times 10^4$ ,  $10^6$  and  $10^{13}$ , respectively.<sup>7</sup> In each of these instances  $Q_{10}$  is constant over the range of lethal temperatures used, but this coefficient evidently is not generally applicable. Bělehrádek (1935) listed temperature coefficients of heat injury for various organisms and tissues, the values of  $Q_{10}$  listed ranging from less than 10 to 58,000. Rapid acclimatization probably accounts for the unusually high values obtained with *Fundulus* and *Atherinops*.

The ranges of temperatures which were tolerated for three days by average 20°-conditioned specimens of the three species studied are 10.4° to 31.7° (21.3° range) for *Atherinops*, 8° to 31.4° (23.4° range) for *Girella* and 3.6° to 35.1° (31.5° range) for *Fundulus*. The 6-day tolerance limits for the *Fundulus*, which are 5.6° and 34.4° (28.8° range), are more nearly comparable with the 3-day values for the other two species. The magnitudes of the ranges are clearly related to the general hardiness of the species, and resistance to cold shows a parallel relationship.

The 20°-conditioned *Atherinops* were about as resistant to heat as *Girella*, but were more sensitive to cooling, their 72-hour lower median tolerance limit being only 1.3° below the lowest weekly average surface temperature recorded near the place of their capture.<sup>8</sup> While the temperature of 20° lies about half way between the extremes of temperature which may be encountered by *Atherinops* in this general locality, and is above the average surface temperature, the specimens taken from 20° withstood for four days a rise of temperature of 11.6° and a fall of only 9.6°. It is concluded that *Atherinops*, like *Girella*, certainly is not any more resistant to cooling than to warming. The importance of lethal cold as a possible limiting factor in the distribution of sensitive marine fishes, and of acclimatization as a factor in their dispersal, has now been amply demonstrated.<sup>9</sup> When all of the pertinent data are considered, it becomes evident that both *Atherinops* and *Girella* almost certainly cannot survive long at 0° even after very gradual cooling, the true range of thermal tolerance for *Girella* being smaller than that plotted by

<sup>7</sup>  $Q_{10} = (K_1/K_2)10/(t_1 - t_2)$ , where  $K_1$  and  $K_2$  are velocity constants at temperatures  $t_1$  and  $t_2$ , respectively, of which  $t_1$  is the higher.

<sup>8</sup> Surface temperatures reported for the northern portion of the reputed range of distribution of *Atherinops affinis* (United State Weather Bureau, 1938) average 11° to 13°, corresponding winter averages being about 1° lower. However, the species may be represented in different portions of its range by physiologically different races, the resistance of which to cold may be inherently different.

<sup>9</sup> For data on the reactions of *Fundulus* and *Atherinops*, as well as of *Girella*, to temperature gradients, which also indicate a pronounced sensitiveness of these fishes to cooling, see Doudoroff (1938).

Brett (1944), who used the author's data, but plotted only 12-hour tolerance limits. The susceptibility of *Fundulus parvipinnis* to chilling is less pronounced, but its ability to withstand 0° indefinitely after slow cooling is questionable, and this species is correspondingly resistant to heat.

There is evidence that not only the speed, but also the cause of death after chilling can vary with the intensity of the cold. The rapid death of *Fundulus* at very extreme low temperatures was quite different from the delayed death which followed more or less prolonged temporary recovery from the initial effects of cooling at less extreme temperatures. Very brief recovery never was observed at intermediate lethal temperatures. Only at the slowly lethal temperatures was death preceded in sea water by evident dehydration of tissues and delayed significantly by dilution of the medium.

The observation that recovery from primary chill-coma could be induced by warming and the administration of artificial respiration when warming alone was not sufficient suggests respiratory inhibition (Weigmann, 1936) as a cause of death at rapidly lethal low temperatures. When respiration is not resumed within a short time after cooling, asphyxiation or carbon dioxide narcosis may prevent subsequent recovery. However, at low temperatures hardy fishes can survive for hours in deoxygenated water (Sumner and Doudoroff, 1938). It is not improbable that asphyxiation was of importance as a cause of death only after warming (i.e., return to a normal temperature) and the consequent increase of the rate of respiratory metabolism.

At slowly lethal low temperatures increasing distress, indicative of cumulative injury, was evident long before the permanent cessation of regular respiratory movements, and the behavior of *Fundulus* did not suggest asphyxiation. It appears that at these temperatures some osmoregulative functions, such as the ingestion of sea water or the active excretion of salts by the gills, which compensate for the passive exosmotic loss of water through the external membranes (Smith, 1930; Keys, 1933), were inhibited, and in the normal medium dehydration and death followed as a result. The failure of these functions probably was itself a consequence of cold injury to tissues involved in osmoregulation (e.g., of the gills, the central nervous system or the integument) and was not, therefore, the primary cause of injury. The fact that mortality, although it was much delayed, was not entirely prevented when dehydration was avoided by dilution of the medium indicates that other, less rapidly lethal disturbances occurred also. Osmoregulative failure is only one of a number of possible causative factors which should be taken into consideration in further studies of the phenomena of chilling.

#### SUMMARY

The resistance of the hardy marine fish *Fundulus parvipinnis* and of the delicate *Atherinops affinis* to low and high temperatures is examined in relation to temperatures of previous acclimatization and to temperature conditions in the natural habitats of the fishes.

The relationships between the time (duration) of exposure to test temperatures and the average (median) limits of temperature tolerance indicate the value of prolonged tests in the study of resistance to cold and heat.

Marked susceptibility of marine fishes to chilling (i.e., injury at non-freezing

low temperatures) and the influence of acclimatization upon temperature tolerance are confirmed.

The death of *Fundulus* at slowly lethal low temperatures, but not at rapidly lethal temperatures, is preceded in the normal medium (sea water) by evident dehydration of the tissues and is delayed by dilution of the medium. Osmoregulative failure is indicated as one of the causes of slow death at low temperatures in sea water, but not of rapid death at more extreme low temperatures, which may be caused partly by respiratory disturbances.

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# THE BIOLOGICAL BULLETIN

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## ORIGIN AND FUNCTION OF THE PROTOPLASMIC CONSTITUENTS IN *PELOMYXA CAROLINENSIS*

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### INTRODUCTION

The protoplasmic constituents of several species of *Pelomyxa* have been described by Greeff (1874), Gould (1894), Wilson (1900) and Wilber (1942) and some suggestions have been made concerning their origin and their function but none of these suggestions are supported by experimental evidence. It, therefore, seems desirable to study the origin and function of these structures experimentally.

### RELATIVE SPECIFIC GRAVITY OF THE PROTOPLASMIC CONSTITUENTS

Open bottom hematocrit tubes were made of pyrex glass. The smaller opening was closed with a rubber band and the tubes were half filled with a heavy solution of gum-arabic in culture fluid. Then about 1 cc. of culture fluid, containing 5 or 10 *pelomyxae*, was carefully added and the solutions at the interface stirred with a glass rod and mixed so as to produce in this region a gradient of density and viscosity. The tubes were then put into an electric centrifuge and rotated, after which the rubber bands were quickly removed and the contents poured into partially frozen culture fluid, so as to prevent recovery of the *pelomyxae*. Some of these centrifuged, chilled *pelomyxae* were put into each of the following: one per cent osmic acid in culture fluid, ninety-five per cent alcohol, Champy's fluid, Regaud bichromate-formol mixture, and Bouin's fluid. These preparations were then studied under high and low magnifications. The results obtained are presented in Figure 1 and the following paragraphs.

Figure 1 shows that the *pelomyxae* during centrifugation were much elongated, that the refractive bodies (Wilber, 1942) aggregated at one end and the fat globules and contractile vacuoles at the other and that the food-vacuoles, the nuclei, the crystals, the beta granules, and the clear hyaloplasm respectively in fairly definite layers between; but it does not show which end is heavier.

Numerous specimens were consequently withdrawn from the tubes immediately after centrifugation, by means of an ice-cold capillary pipette of such small bore

<sup>1</sup> The writer wishes to express his sincere gratitude to Dr. S. O. Mast, who suggested the problem and under whose direction the work was done, for constant encouragement and invaluable aid in preparing the manuscript.

that they could not turn end for end. The capillary was then immersed in nujol, to eliminate distortion caused by the curved side walls, and examined under the compound microscope. Invariably, the refractive bodies were at the centrifugal and the fat globules at the centripetal end. The refractive bodies, consequently,

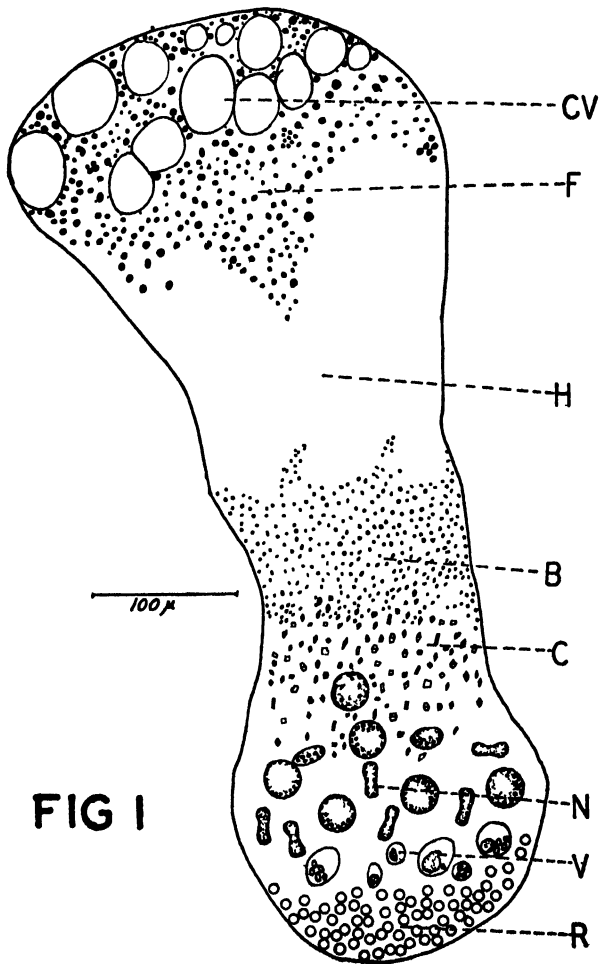


FIGURE 1. Camera sketch of a centrifuged *Pelomyxa* showing stratification of the protoplasmic constituents. R, refractive bodies (at centrifugal end); V, food-vacuoles; N, nuclei; C, crystals; B, beta granules (mitochondria); H, layer containing only hyaloplasm; F, fat; Cv, contractile vacuoles.

are the heaviest and the fat globules and contractile vacuoles the lightest constituents of *Pelomyxae carolinensis*. Mast and Doyle (1935) obtained similar results in observations on *Amoeba proteus*. However the substance in the nuclei did not stratify as in *Amoeba proteus*, indicating that either all the nuclear material has the

same specific gravity or that the viscosity of it is so high that the granules do not stratify under the centrifugation used.

The crystals were free in the cytoplasm, and not in vacuoles as occurs very commonly in uncentrifuged specimens and in centrifuged amoebae (Mast and Doyle), and many of them were broken probably by striking one another during centrifugation. Apparently, at the centrifugation used the crystals were thrown out of the vacuoles and aggregated in a different layer.

#### RECOVERY OF CENTRIFUGED PELOMYXAE

Pelomyxae were centrifuged as described above and put on slides and examined under high magnification while the temperature slowly increased to that of the room. As the temperature increased, the plasmasol (Wilber, 1942) began to flow toward the centripetal end in all but a few specimens in which very small, stubby pseudopods first formed at the centrifugal end but these were soon withdrawn and the plasmasol began to flow in the opposite direction. The refractive bodies were the first protoplasmic constituents to enter the flowing plasmasol. They were carried toward the centripetal end and became distributed in the lower layer of the plasmasol. After the refractive bodies were all in the stream the food-vacuoles were carried forward and came to lie above the refractive bodies; then the crystals and beta granules were carried forward. In this manner the protoplasmic constituents were redistributed through the cell. The pelomyxae remained in the monopodal form during and for some time after the redistribution. The entire process required about 2 minutes.

#### FUNCTION OF NUCLEI

Pelomyxae were mounted on a slide without a cover-slip, examined under the high power of the binocular dissecting microscope, and left undisturbed until they had attached and had extended one or more pseudopods. Then, with the aid of a fine glass needle, pseudopods which contained refractive bodies, crystals, beta granules, and contractile vacuoles, but only a few or no nuclei, were cut off and mounted on a slide under a cover-slip supported by a ring of vaseline, some with numerous chilomonads, others with no food. The slides were examined under the low and high power of the compound microscope to ascertain the effect of removing various numbers of nuclei, on locomotion, feeding, digestion, and longevity.

*Locomotion:* After the operation, most of the fragments remained quiet for 30 to 120 minutes. Then all except those with no nuclei became active and soon moved precisely like normal specimens in all respects, and sudden increase in the intensity of light caused them to stop moving, just as it does normal specimens. Moreover, the method of formation of pseudopods was the same as in normal pelomyxae except that those with less than 9 or 10 nuclei invariably assumed the monopodal form which is not characteristic of uninjured pelomyxae.

All the anucleate fragments were sluggish. Coarsely granular material was aggregated in the center leaving a peripheral layer with no cytoplasmic constituents except beta granules; but usually there was no clear hyaline layer, although some few did have a distinct hyaline cap. The process of locomotion was greatly modified. In fact, most of the fragments did not move at all unless stimulated strongly.

These results are like those obtained by Verworn (1909), Gruber (1912), Stół (1910) and Willis (1916) in observations on *Amoeba*.

**Feeding and Digestion:** Numerous fragments obtained as described above were examined carefully. All nucleate fragments ingested food and the process was the same as in uninjured pelomyxae. Fragments with 1, 3, 6, and 10 nuclei respectively were stained with neutral red and the process of digestion carefully observed. No difference from the normal was evident. Refractive bodies were formed as in normal specimens. The increase in number observed in 36 hours after feeding is given in Table I. This table indicates that the number of refractive bodies formed varied inversely with the number of nuclei.

TABLE I

Table showing the relation between the number of nuclei and the increase in number of refractive bodies in nucleate fragments of *Pelomyxa carolinensis*.

| Number of nuclei<br>in each fragment | Number of refractive bodies |                | Increase in<br>per cent | Average |
|--------------------------------------|-----------------------------|----------------|-------------------------|---------|
|                                      | Before feeding              | 36 hours later |                         |         |
| 1                                    | 10                          | 16             | 60                      | 63      |
| 1                                    | 3                           | 5              | 66+                     |         |
| 3                                    | 9                           | 13             | 44+                     | 45      |
| 3                                    | 4                           | 5              | 25                      |         |
| 3                                    | 6                           | 10             | 66+                     |         |
| 6                                    | 12                          | 17             | 41+                     | 39      |
| 6                                    | 8                           | 11             | 37+                     |         |
| 10                                   | 31                          | 30             | 0                       | 0       |

Ten anucleate fragments were studied. Seven of these did not ingest anything; three ingested one chilomonad each. One of these was observed during the process of ingestion. It formed a food-cup which was apparently normal. The ingested chilomonads died in the food-vacuoles but were not digested. One of the fragments was sketched 24 hours after it had ingested a chilomonad (Fig. 2F). The chilomonad was then dead but still intact and there was no evidence of digestion even after 72 hours. A partially digested chilomonad was in a food-vacuole in this fragment at the time of operation; digestion proceeded no further (Fig. 2F').

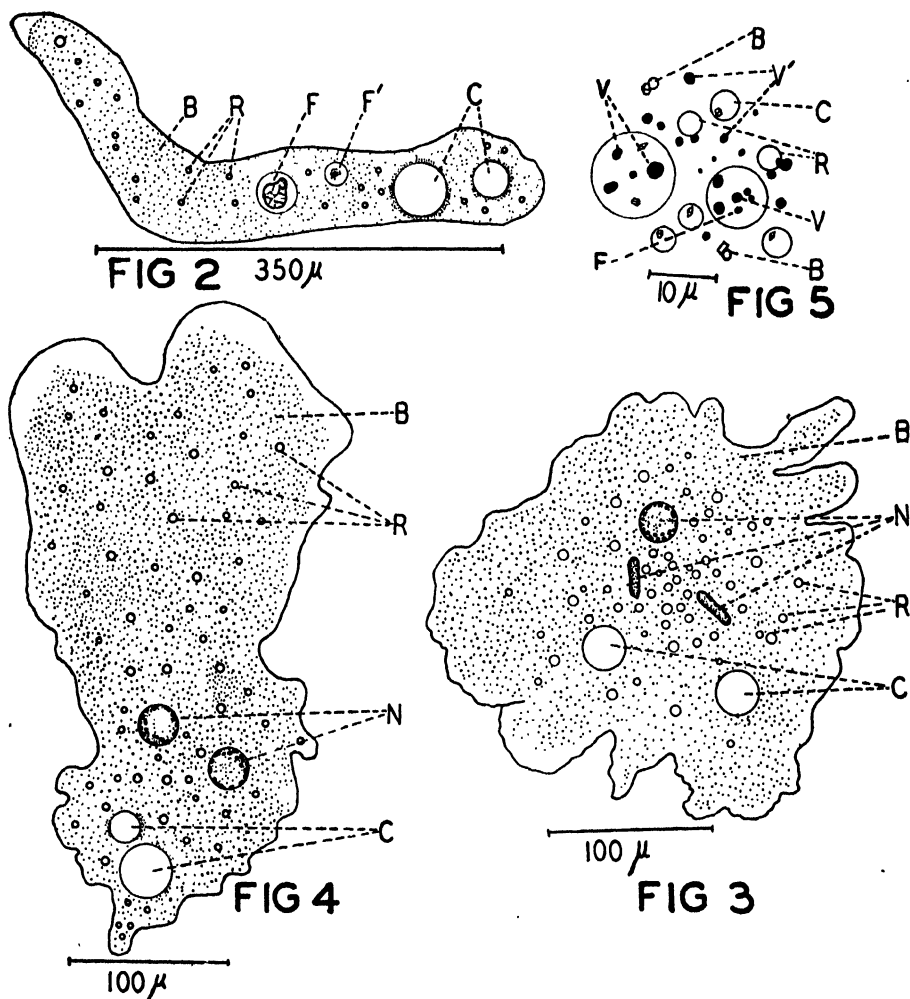
These results indicate that anucleate fragments of *Pelomyxa* sometimes ingest food and kill it but that they do not digest it, or food which was ingested before the operation. The results agree in part with those of Stół (1910) who maintains that anucleate amoebae ingest food normally for several days. They are not in accord with the views of Hofer (1890), Gruber (1912), Willis (1916), Lynch (1919), and Becker (1926) who contend that anucleate amoebae do not ingest food. Becker, however, maintains that in anucleate fragments of *Amoeba dubia* "Food once ingested can be digested by the amoebae after the fashion of normal amoebae."

*Length of life:* Numerous fragments with different numbers of nuclei but with the same number of beta granules, crystals, and refractive bodies (Wilber, 1942) were obtained as described above. Great care was taken to have the volume of the various fragments as nearly equal as possible. They were all passed through five

TABLE II

Table showing the relation between the number of nuclei and the length of life in fragments of *Pelomyxa carolinensis* in the absence of food.

| Number of nuclei | Number of food-vacuoles | Number of refractive bodies | Average | Length of life in days | Average |
|------------------|-------------------------|-----------------------------|---------|------------------------|---------|
| 1                | 0                       | 10                          | 6.2     | 8                      | 6.2     |
| 1                | 1                       | 3                           |         | 4                      |         |
| 1                | 0                       | 5                           |         | 6                      |         |
| 1                | 1                       | 7                           |         | 7                      |         |
| 2                | 2                       | 3                           | 5.6     | 8                      | 6.3     |
| 2                | 0                       | 8                           |         | 6                      |         |
| 2                | 0                       | 6                           |         | 5                      |         |
| 3                | 0                       | 4                           | 7.0     | 5                      | 7.5     |
| 3                | 2                       | 10                          |         | 10                     |         |
| 4                | 1                       | 12                          | 6.0     | 6                      | 8.5     |
| 4                | 2                       | 3                           |         | 10                     |         |
| 4                | 2                       | 3                           |         | 10                     |         |
| 4                | 0                       | 6                           |         | 8                      |         |
| 5                | 1                       | 4                           | 8.0     | 8                      | 8.3     |
| 5                | 9                       | 12                          |         | 7                      |         |
| 5                | 1                       | 8                           |         | 10                     |         |
| 8                | 2                       | 12                          | 11.0    | 19                     | 19.0    |
| 8                | 1                       | 8                           |         | 23                     |         |
| 8                | 3                       | 13                          |         | 15                     |         |
| 10               | 2                       | 14                          | 12.3    | 17                     | 18.6    |
| 10               | 1                       | 12                          |         | 14                     |         |
| 10               | 3                       | 11                          |         | 25                     |         |
| 12               | 1                       | 15                          | 16.0    | 21                     | 20.0    |
| 12               | 3                       | 17                          |         | 19                     |         |
| 20               | 3                       | 20                          | 18.6    | 23                     | 24.0    |
| 20               | 3                       | 19                          |         | 19                     |         |
| 20               | 3                       | 17                          |         | 30                     |         |
| 25               | 2                       | 21                          | 18.5    | 26                     | 23.5    |
| 25               | 1                       | 16                          |         | 21                     |         |
| 40               | 2                       | 16                          | 23.0    | 21                     | 24.0    |
| 40               | 2                       | 30                          |         | 27                     |         |
| 50               | 4                       | 18                          | 22.6    | 27                     | 28.6    |
| 50               | 4                       | 10                          |         | 29                     |         |
| 50               | 3                       | 40                          |         | 30                     |         |



FIGURES 2-5.

FIGURE 2. Camera sketch of anucleate fragment of *Pelomyxa carolinensis*. C, contractile vacuoles; F, food-vacuole containing a chilomonad which was ingested after all the nuclei had been removed; F', food-vacuole present before the operation; R, refractive bodies; B, beta granules.

FIGURE 3. Camera sketch of a fragment of a pelomyxa one minute after it had been cut off. B, beta granules; C, contractile vacuoles; N, nuclei; R, refractive bodies. The fragment had no contractile vacuoles when it was cut off. Note that one minute later, two contractile vacuoles had formed, but that there was no aggregation of beta granules around them. They contracted about one hour after the sketch was made but there was no indication of aggregation even then.

FIGURE 4. Camera sketch of a fragment of a pelomyxa one hour after it had been cut off. N, nuclei; C, contractile vacuoles; R, refractive bodies; B, beta granules. The fragment had no contractile vacuoles until shortly before the sketch was made. Note that a few beta granules

separate 10 cc. portions of sterile culture fluid, then each was put into 5 cc. of this fluid in a small glass dish and observations made on the relation between the number of nuclei and the length of life.

When the fragments failed to respond to stimulation with a fine glass rod, they were tentatively considered dead. When they showed evidence of disintegration, they were pronounced dead. The occurrence of these two phenomena never differed by more than four hours. The results obtained are given in Table II. This table shows that the fragments with one to 5 nuclei lived roughly the same length of time, but that those with more than 5 nuclei lived much longer.

It may be contended that this is due to differences in the number of refractive bodies and food-vacuoles in the fragments or to differences in their size. As stated above, all the fragments were nearly the same in size. The difference in the length of life observed could therefore not have been due to difference in size. The table shows that while in the fragments with 5 or fewer nuclei the length of life usually varied directly with the number of refractive bodies and food-vacuoles, in those with more than 5 nuclei this did not obtain. This seems to show that while the length of life of fragments of *Pelomyxa* is, at least under some conditions, dependent upon the number of refractive bodies and food-vacuoles present, it is also dependent upon the number of nuclei present.

The evidence presented, then, seems to show that the presence of at least one nucleus is necessary for locomotion and digestion but not for ingestion of food, and that the length of life of a fragment, without food, varies directly with the number of nuclei and the number of food-vacuoles and refractive bodies present.

#### FORMATION OF THE CONTRACTILE VACUOLES AND THE RELATION BETWEEN THEM AND THE BETA GRANULES

Numerous pelomyxae were examined under the high power of a binocular dissecting microscope. The animals were left undisturbed on a slide without a cover-slip until they had attached and had extended one or more pseudopods. With the aid of a fine glass rod, pseudopods which contained one or more nuclei, refractive bodies, crystals, and beta granules, but *no contractile vacuoles*, were cut off and mounted on a slide under a cover-slip supported by a ring of vaseline, and examined under the oil immersion objective of a compound microscope.

The following was observed: New contractile vacuoles formed in all the fragments. The time for the formation of new contractile vacuoles varied from about 60 seconds to more than one hour after the operation (Fig. 3). The vacuole was first visible as a small fluid-filled space in the hyaloplasm, no larger than a beta granule. This grew rapidly in size until it was about 5 micra in diameter and then enlarged more slowly until it contracted. At no time was there an aggregation

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have aggregated around them. Ten minutes after this sketch was made, the two vacuoles coalesced and another one formed between the two nuclei.

FIGURE 5. Camera sketch of portion of cytoplasm in a starved pelomyxa twenty-four hours after it had ingested a colpidium. F, food-vacuoles; V, vacuole-refractive bodies in the food-vacuoles; V', vacuole-refractive bodies in the cytoplasm; B, bleb with a crystal attached to it; C, crystal in a vacuole; R, vacuole-refractive bodies in contact with blebs. Note that some of the food-vacuoles contain crystals and vacuole-refractive bodies. Some of the vacuole-refractive bodies are free in the cytoplasm. Several are in contact with blebs formed from the crystals. Note also two crystals with blebs and crystals in vacuoles.

of beta granules in the region where the vacuole formed but after it had discharged a few times, beta granules aggregated around it (Fig. 4).

The time required for the beta granules to aggregate around the contractile vacuole varied greatly. In some of the fragments studied, there was no sign of aggregation for several days after the vacuole had formed and begun to function; in others, aggregation began very soon after it had formed. The presence or absence of nuclei in the fragments had no apparent effect on the time of formation of the contractile vacuole and the variation in time was as great in anucleate as in nucleate fragments, and the nucleus had no effect on the time required for the aggregation of beta granules around the vacuole.

In uninjured pelomyxae, the cytoplasm immediately around the contractile vacuole is more viscous than that which is elsewhere in the plasmasol (Wilber, 1942). Young contractile vacuoles, however, i.e., vacuoles which had recently arisen *de novo*, were seen often to come into intimate contact with nuclei, food-vacuoles, and other structures in the cytoplasm, and the beta granules in the cytoplasm adjoining them had as marked Brownian movement as those elsewhere in the plasmasol. It was not until after the first contraction that evidence of higher viscosity was observed in the cytoplasm adjoining the surface of the newly formed vacuoles. These facts indicate that the cytoplasm adjoining the surface of the contractile vacuole does not become viscous until some time after the vacuole has arisen. After a viscous layer has formed around a contractile vacuole, the latter always expands in this viscous substance after each contraction. New vacuoles, however, arise elsewhere in the cytoplasm.

It thus appears that in *Pelomyxa* the beta granules are not directly related to the formation of contractile vacuoles and that their aggregation and the gelation of the cytoplasm around the contractile vacuoles are in no way necessary for the action of the vacuoles.

These results are in accord with the contention of Mast (1938), in reference to *Amoeba proteus* but not with those of Howland and Pollack (1927) in reference to *Amoeba verrucosa*. Mast says: "The differentiation of a layer of substance on the surface of the contractile vacuole is probably due to the action of the fluid in the vacuole on the adjoining cytoplasm," and "neither the beta granules nor the layer of viscous substance is involved in the function of the contractile vacuole." Howland and Pollack (1927) maintain that the gelated region around the contractile vacuole can "be considered as supplying the initial impulse for systole. . . ." This is not true in *Pelomyxa carolinensis* for in it the gelated layer does not form until after the first contraction. It is, therefore, not necessarily involved in the contraction of the vacuole and it obviously does not necessarily function in the accumulation of fluid in the vacuole.

It can, therefore, be concluded that neither the beta granules nor the viscous cytoplasm around the contractile vacuoles is of primary importance in the formation or the function of the contractile vacuoles.

#### FUNCTION OF THE BETA GRANULES

Wilber (1942) demonstrated that the beta granules in *Pelomyxa carolinensis* stain like mitochondria and he concludes: "These granules, like the beta granules of *Amoeba proteus* (Mast and Doyle, 1935), are consequently similar in composition to the mitochondria in metazoan cells." Metcalf (1910), referring to *Amoeba*

*proteus*, holds that they are involved in the excretion of substance by the contractile vacuole and he consequently calls them "excretory granules."

Mast and Doyle (1935) attempted to ascertain their function in *Amoeba proteus* by centrifuging and then cutting out different proportions and noting the effect and by observing their movements in normal specimens. They concluded that "the beta granules function in accumulation of fluid eliminated by the contractile vacuole." They are the only investigators who approached the problem experimentally.

In the present work, the experiments and observations of Mast and Doyle were repeated on *Pelomyxa carolinensis*. Some specimens were centrifuged and cut at different levels so as to remove various constituents and different proportions of the beta granules (Fig. 1). Others not centrifuged were cut, to obtain fragments of approximately the same size as the centrifuged fragments, but with normal proportions of all the protoplasmic constituents. Both kinds of fragments were mounted in culture fluid, without food, on slides under cover-slips supported by rings of

TABLE III

Table showing the effect of removing different protoplasmic constituents on the length of life of pelomyxae. F, fat; Cv, contractile vacuoles; H, hyaloplasm; B, beta granules.

| Constituents removed         | Number of specimens | Average length of life in days |                         |
|------------------------------|---------------------|--------------------------------|-------------------------|
|                              |                     | Constituents removed           | No constituents removed |
| F + Cv                       | 10                  | 18                             | 21                      |
| F + Cv + $\frac{1}{2}$ H     | 9                   | 7                              | 22                      |
| F + Cv + H                   | 10                  | 1                              | 19                      |
| F + Cv + H + $\frac{1}{2}$ B | 8                   | 1                              | 20                      |
| F + Cv + H + B               | 8                   | $\frac{1}{2}$                  | 15                      |

vaseline and studied until all the fragments had died. This was repeated five times. The results obtained in a typical experiment are presented in Table III.

Table III shows the following: The uncentrifuged fragments lived, on an average, from 15 to 22 days. The centrifuged fragments produced by cutting so as to remove the fat and contractile vacuoles lived nearly as long as the uncentrifuged fragments. Those produced by cutting so as to remove the fat, contractile vacuoles, and about half of the hyaloplasmic layer lived about one-third as long. Those produced by cutting so as to remove practically all the hyaloplasmic layer lived only about one-twentieth as long, but those produced by cutting so as to remove approximately half of and all the beta granules respectively showed no marked further decrease in length of life.

It is evident from these results that the removal of the fat and the contractile vacuoles causes but little, if any, decrease in length of life, that removal of a large proportion of the hyaloplasm, in addition to the fat and the contractile vacuoles, causes a pronounced decrease in the length of life, and that the removal of the beta granules in addition to the hyaloplasm, fat, and contractile vacuoles causes little, if any, further decrease in length of life. These facts indicate that length of life in fragments of *Pelomyxa* is primarily dependent on the amount of hyaloplasm in them, and only secondarily, if at all, on the number of beta granules.

The results are in harmony with the contention of Holter (1936) and Holter and Kopac (1937) that enzymes in amoebae and in marine ova are localized in the hyaloplasm and not in any formed bodies, and also with the views of Just (1939) in regard to the great importance of the ground cytoplasm in cellular function.

The facts presented, therefore, seem to indicate that beta granules (mitochondria) do not play an active part in cell functions and that their observed movements are merely a visible sign of submicroscopic changes in the hyaloplasm.

#### FORMATION OF REFRACTIVE BODIES

About 100 pelomyxae were centrifuged and cut so as to remove most of the fat and all the refractive bodies (the fat was removed so that it could not serve as a source of food). Half of them were then put into each of two dishes containing sterile culture fluid and numerous colpidia added to the fluid in one of the dishes. In about 50 minutes all the specimens had recovered from the "shock" of the operations, as indicated by the facts that they moved normally and responded to increase in intensity of light by cessation of movement.

Two of the 50 pelomyxae without food died 20 hours after the operation; four of them lived until the 7th day; none divided. The average length of life was five days. No vacuole-refractive bodies (Wilber, 1942) or refractive bodies formed in any of them. Three of the 50 pelomyxae with food did not feed and died four hours after the operation. Each of the others ingested one or more colpidia. Twenty-four hours after feeding, there were numerous vacuole-refractive bodies in the food-vacuoles and some in the cytoplasm of every one of these specimens, and some of the food-vacuoles in a few of them contained crystals. These results show that food is necessary for the formation of vacuole-refractive bodies and of refractive bodies, and that the crystals are formed in the food-vacuoles. They also show that removal of the refractive bodies has no apparent injurious effects on pelomyxae. This is in accord with the views of Mast and Doyle (1935) who contend "that the removal of all the refractive bodies does not seriously interfere with the vital processes in *Amoeba*," but is not in accord with the views of Singh (1938) who maintains that specimens of *Amoeba proteus* with the refractive bodies removed appear "as if they were encysted until these bodies are formed," and that "as soon as these bodies are formed, the amoebae regain their normal shape and activity."

Ten pelomyxae were kept in sterile culture fluid for 15 days; then colpidia were added and each pelomyxa examined under low and high magnifications at intervals of one hour. The results obtained in observations on all these pelomyxae are in accord with the following detailed description concerning one which had ingested two colpidia: One hour after ingestion, the two colpidia were dead, but there was no evidence of digestion. Two hours after ingestion, both colpidia stained deeply red with neutral red, but the fluid in the food-vacuoles did not stain; the vacuolar membrane was very close to the pellicle of the colpidia. Three hours after ingestion, the colpidia stained deeply red in neutral red, and the fluid in the vacuoles, orange; the vacuoles had enlarged, probably as a result of fluid passing in from the cytoplasm. Four hours after ingestion, both food-vacuoles had divided; fragments of the colpidia in the four resulting vacuoles stained deeply red; two of the vacuoles contained vacuole-refractive bodies which stained intensely red in neutral red (one

vacuole had three; the other, one). Six hours after ingestion, there were six food-vacuoles present; all contained vacuole-refractive bodies; two contained hyaline nonstaining spherical bodies about 4 micra in diameter and of unknown composition. Twelve hours after ingestion, there were numerous vacuole-refractive bodies in each of the six vacuoles and several crystals in four of them; the other two contained no crystals; the vacuole-refractive bodies varied from 1 micron to about 6 micra in diameter; several were free in the cytoplasm; the manner in which they entered the cytoplasm was not ascertained. Twenty-four hours after ingestion, there were eight food-vacuoles present; five contained vacuole-refractive bodies and crystals, three, only vacuole-refractive bodies; there were numerous vacuole-

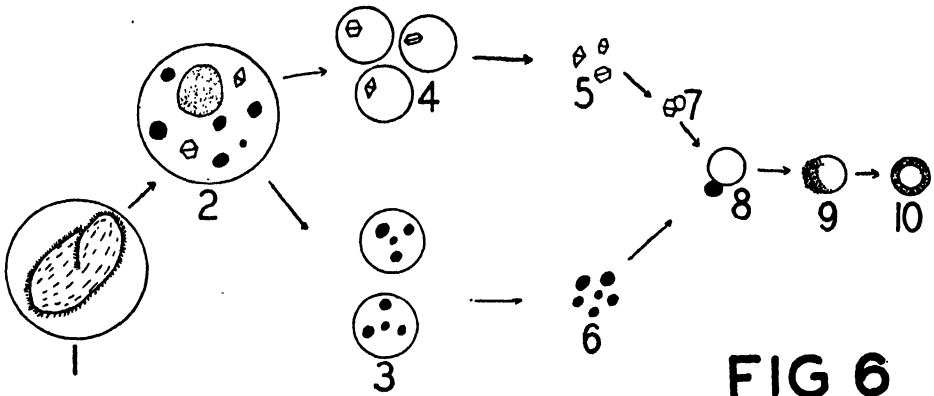


FIGURE 6. Diagram illustrating the formation of the refractive bodies from digested food. 1, food-vacuoles containing an ingested organism, usually a colpidium or a chilomonad; 2, food-vacuole containing a partially digested organism and crystals and vacuole-refractive bodies formed from the food during the process of digestion; 3 and 4, vacuoles formed by the division of 2; 5 and 6, crystals and vacuole-refractive bodies free in the cytoplasm; the manner in which they leave the vacuoles is not known; 7, crystal with bleb attached to it (the crystal disappears and the bleb greatly enlarges); 8, enlarged bleb in contact with a vacuole-refractive body; 9, vacuole-refractive body "flowing over" the bleb; 10, refractive body formed by the union of a bleb and a vacuole-refractive body. Stippling in 9 and 10 represents material of vacuole-refractive body.

refractive bodies and several crystals in the cytoplasm; some of the crystals had blebs attached (Fig. 5).

One of the crystals with a bleb attached was observed closely under oil immersion for several hours. The bleb became larger and simultaneously the crystal smaller until it was no longer visible. Soon another crystal came in contact with the bleb, and in about 40 minutes it too disappeared and the bleb became still larger. The crystals were obviously used in the growth of the bleb. Fifteen minutes later, the bleb came in contact with three vacuole-refractive bodies which were free in the cytoplasm. The substance in these three bodies "flowed over" the bleb and covered it with a layer, resulting in the formation of a body which was identical with other refractive bodies. The time required was 65 minutes. The beta granules took no visible part in the formation of the refractive body. Similar results were obtained in observations on crystals with blebs attached and vacuole-refractive bodies in normal pelomyxae.

The present observations clearly indicate that both the crystals and the vacuole-refractive bodies originate from the food in the food-vacuoles, that they leave the vacuoles and are found free in the cytoplasm (the manner in which this takes place is not known), and that the crystals form nonstaining spherical bodies (blebs) which become covered with a layer of substance from the vacuole-refractive bodies and thus form refractive bodies (Fig. 6).

The conclusions concerning the formation of the refractive bodies from the crystals and the vacuole-refractive bodies are in accord with the contention of Mast and Doyle (1935) who maintain that "The refractive bodies . . . originate and develop in the cytoplasm probably from substances obtained from the vacuole-refractive bodies and the crystals."

Mast and Doyle (1935) maintain that the beta granules function "in transferring digested substances from the food-vacuoles and the crystal vacuoles to the refractive bodies." There is no indication of such a function in *Pelomyxa*.

#### FUNCTION OF THE REFRACTIVE BODIES

Fifty pelomyxae were passed through five different portions of sterile culture fluid and then put into a stender dish containing 25 cc. of this fluid but no food. Each day a few were stained with neutral red, Sudan black, and Janus green B respectively, and these and a few unstained specimens examined for refractive bodies, fat, beta granules, and crystals. The results are presented in Table IV.

TABLE IV

Showing the decrease in number of refractive bodies in the absence of food.

| Date     | Number of refractive bodies in each of 9 pelomyxae |    |    |    |    |    |    |    |    | Average |
|----------|--|----|----|----|----|----|----|----|----|---------|
|          | a  | b  | c  | d  | e  | f  | g  | h  | i  |         |
| Sept. 25 | 50   | 20 | 40 | 50 | 62 | 31 | 23 | 41 | 40 | 39.7    |
| Sept. 30 | 42   | 18 | 32 | —  | 50 | 27 | 17 | 34 | 30 | 31.2    |
| Oct. 1   | 30   | 10 | 30 | 33 | 50 | 26 | 13 | 30 | 30 | 28.0    |
| Oct. 2   | 23   | 10 | 27 | 27 | —  | 26 | 13 | 30 | 23 | 22.3    |
| Oct. 3   | 20   | 8  | 27 | 27 | 43 | —  | 11 | —  | 23 | 22.7    |
| Oct. 4   | 17   | 8  | 23 | 24 | 43 | 23 | 11 | 25 | 20 | 21.5    |
| Oct. 5   | 14   | 8  | 20 | 20 | 41 | 21 | 11 | 25 | 19 | 19.8    |
| Oct. 10  | 14   | 8  | 17 | 20 | 37 | 15 | 7  | 21 | 10 | 16.4    |

This table shows that there is in *Pelomyxa carolinensis* a steady decrease in the number of refractive bodies during starvation. It was observed that some of the refractive bodies disintegrated and that during disintegration the outer layer gradually disappeared, leaving a homogeneous mass which did not stain neutral red. These results indicate that the refractive bodies function as reserve food. This conclusion is in accord with that of Mast and Doyle (1935) and Singh (1938) in regard to the function of the refractive bodies in *Amoeba proteus*.

#### SUMMARY

1. Centrifugation causes the protoplasmic constituents of *Pelomyxa carolinensis* to stratify into definite layers which, in order of decrease in weight, are as follows:

refractive bodies, food-vacuoles, nuclei, crystals, beta granules, hyaloplasm, contractile vacuoles, and fat.

2. The presence of at least one nucleus is necessary for normal locomotion and digestion, but not for ingestion of food in *Pelomyxa*. The length of life of *Pelomyxa* during starvation varies directly with the number of nuclei present.

3. The contractile vacuoles arise *de nova* in the hyaline cytoplasm. Their formation and functioning is not dependent on the presence of nuclei or beta granules.

4. The beta granules (mitochondria) do not play an active role in cell functions but, probably, merely give visible evidence of submicroscopic changes taking place in the hyaloplasm.

5. The vacuole-refractive bodies and the crystals are formed from the food in the food-vacuoles of *Pelomyxa* and are used in the formation of the refractive bodies.

6. The refractive bodies function as food reserve in the cell. Their complete removal does not impair any of the cellular activities.

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# STAGES IN THE DEVELOPMENT OF THE PICKED OR SPINY DOGFISH, *SQUALUS ACANTHIAS* LINN.

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## INTRODUCTION

In a previous paper, von Bonde (1945), I described various features of the external development of the oviparous Banded Dogfish, *Haploblepharus edwardsii*. The present species, which attains a length of 915 mm., belongs to the group whose development is classified as ovoviviparous. This species (Plate I, Fig. 1) is very common in the Cape and Natal waters of South Africa and its distribution ranges along the Atlantic coasts of Africa, Europe, and North America and to the Mediterranean.

An egg-case containing three developing embryos (Plate II) was laid by a female in one of the tanks of the Sea Point Aquarium.

## FEMALE REPRODUCTIVE ORGANS

The only secondary external sexual characters in the female are the pair of cloacal papillae situated one on either side of the median line immediately posterior to the cloacal aperture. The female contains a paired ovary situated in the usual position towards the anterior end of the coelom. These ovaries (Plate I, Fig. 2, r.ov., l.ov.) change in shape and size according to whether the female is gravid or not, although the cycle of the ovarian growth of the ova is such that ripe ova are again in evidence immediately after the young are born. The paired oviducts are not so highly differentiated in structure and function in their anterior half as those of *Haploblepharus edwardsii*, since the ovum or ova is usually enclosed in a very thin transparent chitinous capsule which is not as highly specialized as the egg capsules or mermaid's purses of the oviparous species. The posterior half of each oviduct, as will appear later, is much more highly developed and specialized in conformity with the ovoviviparity of this species. The oviducts meet in the middle line at the anterior end of the coelom and open into the body cavity by a single orifice. By making a transverse incision immediately posterior to the edge of the pectoral girdle and carrying this incision laterally and then anteriorly and lifting the ventral muscular wall, the confluence of the oviducts is exposed. This common opening of the oviducts is attached by a mesentery in the middle line to the surface of the ventral wall of the coelom (Plate I, Fig. 4, f.t.o. and m.). This common opening is wide and funnel-shaped and lies right over the antero-ventral part of the ovaries so that immediately after a ripe ovum escapes from the ovary it is taken up by the lips of the funnel and passed backward along the fallopian tube (Plate I, Fig. 4, f.t.).

Each oviduct may be divided into five distinct portions as follows:

(1) The fallopian tube (Plate I, Figs. 2 and 4, f.t.) is extremely narrow, thin-walled and comparatively short. This portion is followed by (2) the albumen gland (a.g.) which in this species is very small, thick-walled and muscular, the internal surface being richly supplied with glands secreting the albumen which covers the ovum. The albumen gland is followed by (3) the nidamental gland (n.g.) which is a long thin tube of about the same diameter as the fallopian tube. Here the transparent chitinous egg capsule is secreted. The nidamental gland opens posteriorly into (4) a very wide saccular uterus (u.) in which the development of the embryo takes place. The uterus in turn narrows into (5) a short vagina (v.) which opens into the cloaca. In immature females the vaginal orifices are separated from the cloaca by a membrane or hymen which during copulation is pierced by means of the special claws of the claspers (see below). It is interesting to note that in those Elasmobranchs in which the shape of the claspers of the male is flat, the vaginal orifices are slit-shaped, while in those forms in which the claspers are provided with hooks, the lining of the vagina is thickened (Daniel, 1934).

The uterus, which was described in detail by Widakowich (1907a), is more or less typical of those species displaying viviparity or ovoviviparity. In *S. acanthias* each uterus is a greatly enlarged sac with well defined boundaries. The mucous membrane of each uterus varies greatly in conformity with the development of the ovaries. Thus, when the ovaries are large and contain ripe ova ready for fertilization, the mucous membrane of the uterus is more or less smooth. In gravid females, on the other hand, this mucous membrane is thrown into longitudinal rows of flaps known as papillae or villi, each of which is a leaf-like structure with very thin walls (Plate III, Fig. 10, p.). The border of each papilla is thickened by a blood vessel. The blood supply to the uterus is exceedingly profuse (Daniel, op. cit.). The arterial supply consists in part of the anterior and in part of the posterior oviducal arteries which break up into branches to the rows of papillae. Each terminal artery runs along the free borders of the papillae to supply each single papilla with blood. The blood from each papilla is drained by central veins into a main uterine vein. The two systems are connected by a capillary net around the surface of the papilla.

Ercolani (1879) described the viviparous Selachians according to the various aspects of the relationships between the developing eggs and the inner wall of the uterus, and, according to the degree of these relationships, he differentiated four classes:

1. Naked contact between the homogeneously smooth mucous membrane of the uterus and the surface of the egg (*Plagiostomi acotyledonale*). *Squatina angelus* and *Heptanchus cinereus* are cited by him as examples.

2. Complicated contact between both surfaces, the complication being due to the presence of numerous folds in the secreting mucous membrane of the mother which causes an appreciable increase of the inner surface (*Plagiostomi acotyledonale*). *Squalus acanthias* and *Scymnus lichia* are cited as examples.

3. The complicated contact is differentiated by a more or less significant new structure of papillae on the folds of the secreting mucous membrane of the uterus by means of which its inner surface and activity are extraordinarily enlarged (*Alcuni selachii*). *Torpedo* and *Pteroplatea micrura* are cited as examples. (Note: According to the development of the papillae in the uterus it appears as though *S. acanthias* should be placed in this group rather than in Group 2.)

4. The relationship is no longer due to contact but to an intimate union between a part of the absorbing upper surface of the egg with a part of the secreting surface of the uterus. Both surfaces form folds which interlace and at the same time fuse with each other. Thus a type of rudimentary placenta is formed which is comparable with the mammalian placenta (*Plagiostomi cotyledonale*). *Carcharias glaucus* and *Mustelus laevis* are cited as examples.

In *S. acanthias* the papillae of the mucous membrane arise from a cutaneous development of the convoluted marginal arteries. The papillae are twisted around their longitudinal axes. The changing form of the papillae is due partly to a purely mechanical and partly to a reflex action. There appears to be no relationship between the gestation period and the length of the papillae. The uterus is supplied with blood from two capillary systems, viz., (a) the organotropic and (b) the embryonotropic. The latter forms a capillary network which covers the papillae and also the remaining part of the inner wall of the uterus. The capillary network receives its blood from the marginal arteries and releases it into the basal veins. These do not possess any separate musculature but are embedded in the muscular fasciculi of the uterus which open towards the papillae.

Widakowich (1907b) described the closing device ("Verschlussvorrichtung") situated between the portion I have designated as the nidamental gland and the dome of the uterus (Plate I, Fig. 2A, 2B, c.d.). The gravid uterus (Plate I, Fig. 2, u.) has a cylindrical shape with conical ends pointing anteriorly and posteriorly. The uterine arteries are strong walled (Plate I, Fig. 2A, a.) and run laterally along the nidamental glands. The uterine vein (ve.) runs in close proximity to the artery. The closing device is necessary since the uteri, as soon as the embryos reach a definite age and their enveloping membranes rupture, are filled with a large quantity of nutrient yolk, which, but for this closing device, would be forced along the whole length of the oviducts during any contraction of the uterus, or external pressure from the distension of the alimentary channel, or the movements of the embryos in the uteri should the yolk sac be ruptured. During the time of rut and fertilization there is a strong hyperaemia of the whole genital tract; hence the genital tract is well supplied with blood vessels. The function of the papillae above referred to will be dealt with later.

#### MALE REPRODUCTIVE ORGANS

The morphology of the male reproductive system follows the general plan of all Elasmobranchs. The testes are paired and occupy the anterior half of the

#### PLATE I

FIGURE 1. Left lateral view of *Squalus acanthias*.

FIGURE 2. Dissection of a gravid female showing the reproductive organs.

FIGURE 2A. Closing device (c.d.) in oviduct (after Widakowich).

FIGURE 2B. Internal surface of the dome at the anterior end of the uterus showing the closing device. The dome has been invaginated to bring the closing device into prominence, hence it appears nipple-shaped.

FIGURE 3. Egg capsule containing single ovum in early stages of development.

FIGURE 4. Portion of the anterior ends of fallopian tubes (f.t.) showing their confluence and coelomic orifice (f.t.o.).

FIGURE 5. Clasper of male.

FIGURE 6. Claw (cl.) and spur (sp.) of clasper greatly enlarged.

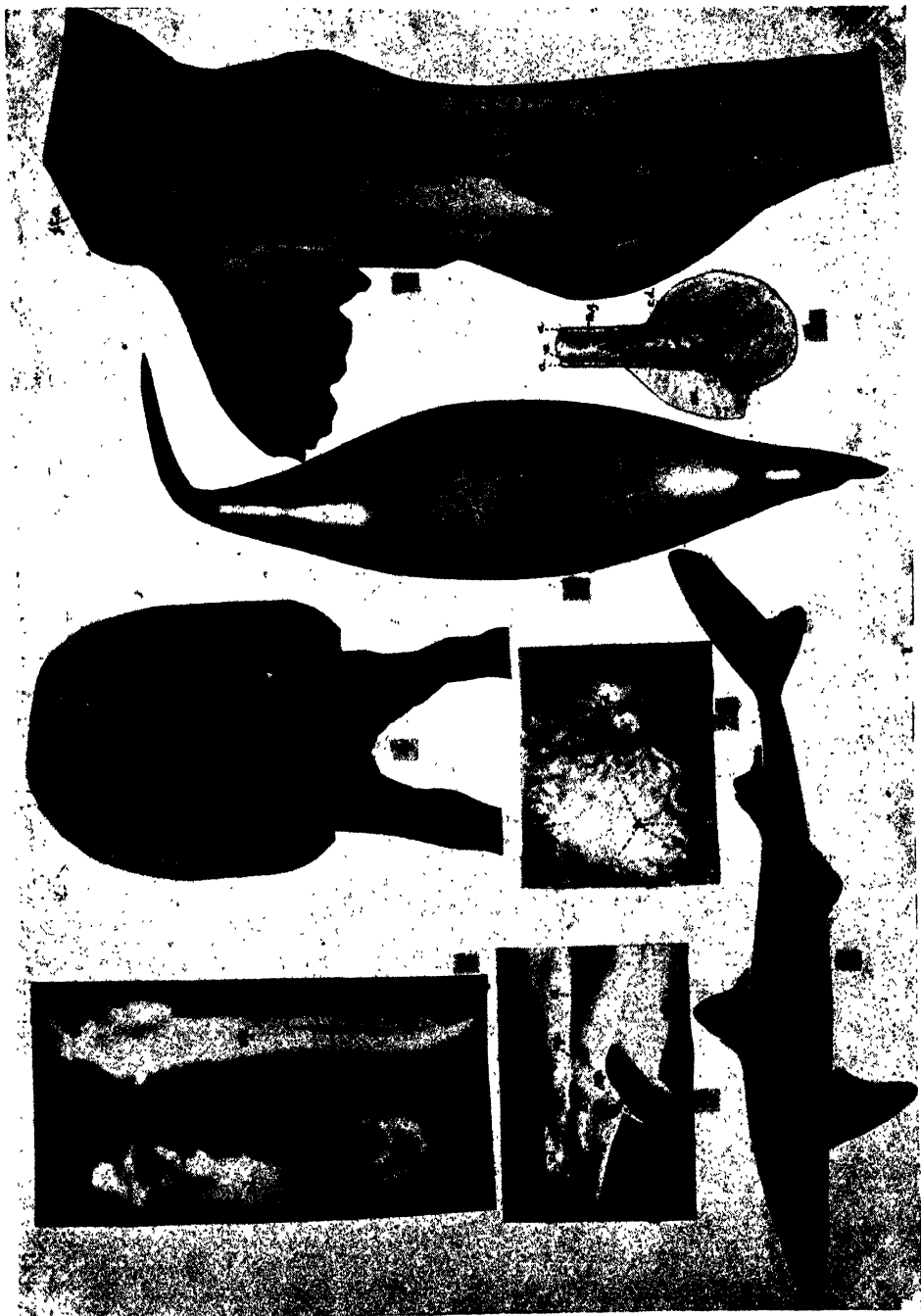


PLATE I

coelom. From the anterior end of each testis the vasa efferentia lead to the epididymus which is much coiled. The epididymus eventually widens out into the vas deferens which expands and joins its fellow in the middle line posteriorly, opening into the cloaca by a single pore.

The external secondary sexual characters of this species present certain distinctive features. As in all Elasmobranchs the basal element (basipterygium) of each pelvic fin is prolonged as a stout, backwardly-directed rod, sharply demarcated from the remainder of the fin, forming an intromittent organ, called the clasper or myxopterygium. A transverse section of the proximal half of the clasper shows it to be almost completely round, the internal cartilaginous skeleton forming a tube filled with a thick muscular substance through the center of which passes a duct. In the distal end the skeleton only occupies the ventral part of the clasper, being continued right to the tip. The latero-dorsal wings of this part of the clasper are formed of thickened skin, the outer wing being folded over the curved inner wing to form a canal which is continuous with the above-mentioned duct in the proximal half. This duct opens through the hypopyle into an open channel which is continued to the tip of the clasper (see Plate I, Fig. 5, hy.). On the outer surface near the hypopyle, a strong spiny spur (sp.) arises. On the inner ventral surface a claw (cl.), which extends to the tip of the clasper, is situated. This claw is much curved and, like the spur, is movable. Its function is probably to rupture the hymen. By pressing the distal end of the clasper, the spur is erected, and forms an important organ of attachment, penetrating and even lacerating the tissues of the female (Leigh-Sharpe, 1920). The spur in its retracted position lies alongside the claw in the same groove. The rhipidion (r.) in the normal intromittent position of the clasper covers the dorsal part of the groove and also the claw and the spur. The siphon (s.) is comparatively small and is situated immediately beneath the skin of the pelvic fin. The apopyle (ap.) lies some distance from the cloaca and relatively near to the hypopyle.

#### DEVELOPMENT

It was not possible to study the complete development of the present species along the lines described in my previous paper (op. cit.) and it is, therefore, necessary to refer to the description of embryos of various lengths taken from gravid females. The gravid uteri shown in Plate I, Fig. 2, u. each contained a single egg capsule with a single ovum (Fig 3.), one being infertile.

A brief description of the primary phases in Selachian development is as follows: At the time of fertilization the ovum is usually in the shape of a large spherical mass, similar to the yolk of an avian ovum, but its shape may become roughly cylindrical as in the present case, the shape being determined by that of the enclosing egg capsule. The greater part of the egg is formed of peculiar oval spherules of food-yolk held together by a protoplasmic network (Balfour, 1881). The yolk is completely covered by the albumen which is thick and glairy and transparent except at the polar extremities. The protoplasm is concentrated in a small lens-shaped area, the germinal disc or "orange spot," and is situated much nearer to the broader end of the egg. Yolk spherules are also present in this disc, but are much smaller and of a different character. Segmentation is meroblastic or discoidal as in all telolecithal eggs, being confined to the germinal disc.

The blastoderm is thus formed and at the close of segmentation the blastoderm forms a lens-shaped disc thicker at one end than the other, the thicker end being known as the embryonic end where the future embryo develops. The three germinal layers become established and shortly after this the rudimentary embryo consists of an oblong plate which extends inwards from the periphery of the blastoderm. This plate is the medullary plate and along its axial line a shallow groove, the neural groove, is formed. The embryo now progressively increases in length and a certain number of mesoblastic somites become formed. The increase in length of the body takes place normally by growth in the region between the last mesoblastic somites and the end of the tail. The anterior part of the body is not completely folded off from the blastoderm and the neural groove becomes converted into the neural canal. As soon as the embryo has become folded off from the yolk both in front and behind, the separate parts of it are easily recognizable. The embryo is attached to the yolk by a distinct stalk, which gradually narrows and elongates and is known as the umbilical cord. The blastoderm continues to grow in all directions and by circumscrescence it provides a protective vascular covering of the yolk mass, but these extra embryonic structures will eventually be resorbed. The protective covering is developed from the ectoderm, whilst the vascular covering arises from the mesoderm. Before completely covering the yolk mass, however, at a certain stage a yolk blastopore, which is that portion of the surface of the yolk mass not yet covered by the blastoderm, is formed. The yolk blastopore is related to gastrulation since circumscrescence is an important factor in gastrulation and it assists in laying down the definitive endoderm. On the ventral surface of the head of the embryo the stomodaeum is formed, which eventually gives rise to the mouth, while some little distance from the posterior end of the embryo the proctodaeum will eventually give rise to the anus. At a somewhat later stage the gill clefts are formed, being at first six pairs in number, but soon the anterior pair becomes separated from the rest to give rise to spiracles. The first rudiment of the heart becomes visible during the 38 somite stage and has a cavity situated between the mesoderm and the endoderm. All the visceral clefts, including the spiracles, show long filiform external gills which project out for some distance from the sides of the head. These external gills lie against the front wall of the spiracle and the front walls of the visceral clefts. It appears as though they are specially developed to facilitate respiration during the earlier stages of development, either intra-uterine or intracapsular. They disappear some time before the close of the embryonic stage. The young *Selachian* has all the external characters of the adult when hatched, and can immediately swim about and fend for itself, but before hatching, the yolk has been completely absorbed by the developing embryo and the yolk sac and umbilical cord decrease in size until eventually they are only shown in the form of an umbilicus which persists for about 14 days after birth. The vitelline circulation becomes developed at a stage before the closure of the yolk blastopore. During the circumscrescence of the blastoderm its edge has remained thickened and this feature persists until the whole of the yolk is covered. In this thickened edge a circular vein arises which brings the blood back from the yolk sac to the embryo. The first stage in the development of the vitelline circulation is the formation of a single artery which emerges from the umbilical cord and proceeds cephalad along the blastoderm under cover of the head of the embryo. This arterial trunk divides to form two arcuate branches that turn laterad and then posterad towards the posterior margin

of the blastoderm. These arterial branches eventually meet posteriorly and give off numerous small secondary branches, some of which connect with small veins emptying into a venous ring close to the margin of the blastoderm. The main trunk of the vitelline vein drains the venous ring and runs straight to the umbilical cord (Smith, 1942). When the yolk blastopore has completely closed, the venous ring disappears and the area formerly occupied by the yolk blastopore is traversed by a continuation of the main trunk of the vitelline vein. This trunk receives numerous small veins usually joining it at right angles. Since the venous trunks are only developed behind the embryo, it is only the posterior part of the arterial ring which gives off branches. At a still later stage the arterial ring embraces the whole yolk, but later vanishes in its turn, as did the venous ring before it. There is then present a single arterial trunk and a single venous trunk. The arterial trunk is a branch of the dorsal aorta, and the venous trunk enters the heart together with the splanchnic vein. The venous trunk enters the body on the right side and the arterial leaves on the left of the umbilical cord.

The yolk sac persists during the whole of the embryonic life and in the majority of Elasmobranch embryos there arises within the body walls an outgrowth from the umbilical canal into which a large amount of the yolk passes. This outgrowth forms an internal yolk sac. In viviparous species a remarkable peculiarity arises in that the vascular surface of the yolk sac becomes raised into a number of folds which fit into corresponding depressions in the vascular walls of the uterus. The yolk sac becomes in this way firmly attached to the walls of the uterus and the two together constitute a kind of placenta (Balfour, op. cit.).

We may now proceed to a description of the various embryos obtained from gravid females:

1. The earliest stage found is that shown in Plate I, Figure 3. The large ellipsoidal yolk mass, having an axial length of 58 mm., is shown enclosed in the transparent egg capsule which had an overall length of 100 mm. The oval shaped blastoderm, measuring 29 mm. along its longer axis, lies on the posterior half of the yolk and at the rounded anterior end it shows the formation of the neural groove (ne.g.). The thickened edge of the blastoderm (b.e.) is well shown. The egg capsule is composed of an extremely thin membrane which is wholly transparent except at the polar regions where the capsule tapers to a sharply pointed end posteriorly and a blunter pointed end anteriorly. These ends are designated anterior and posterior in relation to the position of the embryo and not in regard to the position the capsule takes in the gravid uterus. The figure shows the capsule in the position in which it lies in the uterus, the blunter anterior end being directed posteriorly in relation to the female. The yolk has a thin covering of glairy albumen over the whole surface, but towards the poles the albumen is much thicker and becomes more or less opaque and is loosely attached to the capsule. Encapsulated eggs, which were removed from the uteri, were placed in a large pneumatic trough with running sea water. After a few days the capsules invariably ruptured around the equator and albumen was only found in the pointed polar ends. Compared with the egg cases of oviparous species, the egg capsules of *S. acanthias* contained very little albumen.

2. Plate II shows an egg capsule containing developing triplets (em. 1, 2, 3). These embryos were about 40 mm. in length, the total length of the capsule being

180 mm. The yolk was rounded in conformity with the shape of the enveloping capsule. All attempts to keep these embryos alive after the extrusion of the capsule failed. Embryo 2 ruptured its yolk sac and died, the remaining two embryos also succumbing in rapid succession.

3. The next stage is shown in Plate III, Figures 7A and B. These embryos were taken from a gravid female which had a single egg capsule in each uterus, each capsule containing a set of twins. Immediately after the female was opened, the capsules were removed from the uteri and placed in running sea water in a pneumatic trough. It was not possible to determine their age at this stage. After three days, one capsule ruptured and the embryos freed themselves from their enveloping membrane and lived for about twelve days. The twins in the other capsule died after five days and it is interesting to note that then they were smaller than their counterparts, although at the time of removal from the uteri, all the embryos were of equal size, viz., 45 mm. in length. The longitudinal diameter of the yolk sac at this time was 80 mm. The live embryos, a week later, had attained a length of 65 mm. The longitudinal diameter of the yolk sac was now 70 mm., thus showing

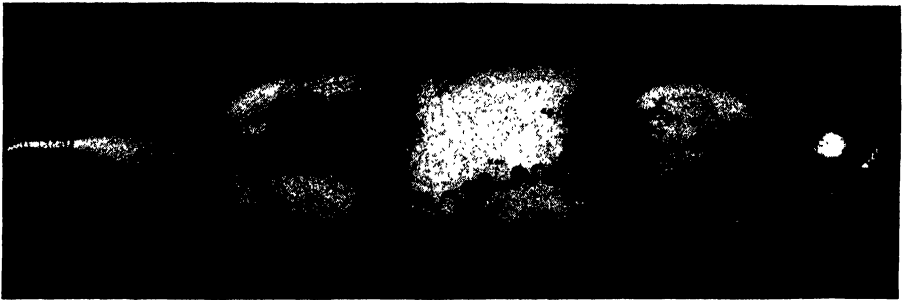


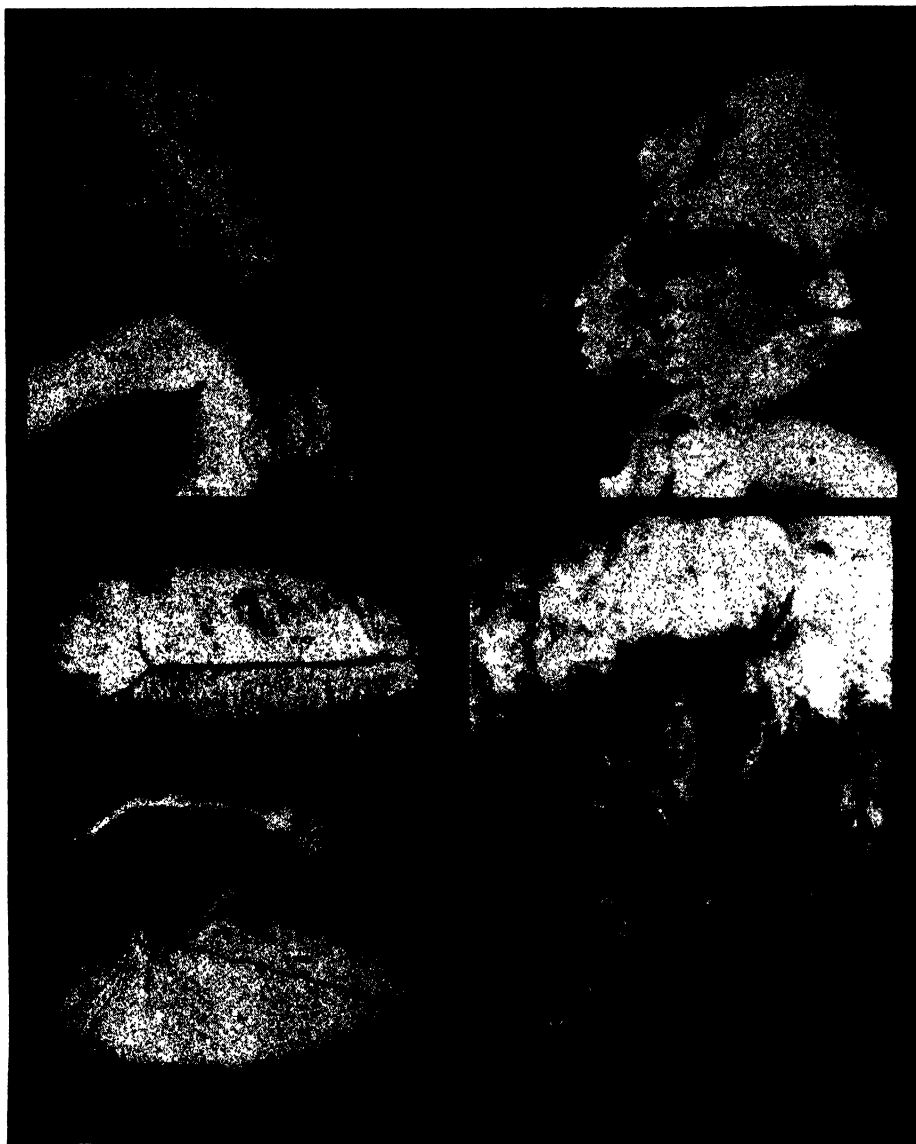
PLATE II

Egg capsule containing three developing embryos.

a corresponding decrease as the embryo grows. The umbilical cord (u.c.) was relatively long and hollow, forming a tubular connection between the yolk sac and the intestine of the embryo. By a gentle pressure on the yolk sac it was possible to force particles of yolk along the umbilical cord into the embryo's intestine. Its walls were very thin and laterally in them ran the vitelline vein (v.v.) and artery. The internal anatomy was well developed at this stage, the alimentary canal being completely formed with a spiral valve developed in the valvular intestine. The liver was developed and the heart had assumed the typical Selachian form. The external gills (Figs. 8 and 9, e.g., sp.g.) had reached their maximum development. After twelve days the other pair died and the external gills were then beginning to shrivel up.

4. A more advanced embryo is shown in Plate IV, Figure 12. Here the external gill filaments had disappeared. The yolk sac was now relatively small and the umbilical cord short and thick. The embryo had assumed the typical adult form except for the yolk sac.

5. The most advanced stages of development found in a gravid female occurred



### PLATE III

FIGURE 7A. A 70 mm. embryo showing the vitelline circulation and the attachment to yolk by the umbilical cord (u.c.).

FIGURE 7B. The hemisphere opposite to the one to which this embryo is attached.

FIGURE 8. Cephalic end of the same embryo showing the external gill filaments (e.g.) and the spiracular gill filaments (sp.g.).

FIGURE 9. Dissection of the buccal cavity of the same embryo showing the external gill filaments attached to the visceral arches (v.a.). The internal branchial clefts (i.br.c.) are also shown.

FIGURE 10. A portion of the internal surface of the uterus, much enlarged, showing the papillae (p.).

in a female 800 mm. in length. Here the six embryos were at the point of birth. They were 220 mm. in length and differed from the one shown in Plate IV, Figure 12 principally in the fact that the yolk had been completely resorbed and the re-



PLATE IV

FIGURE 11. Entire embryo of 100 mm. attached to the yolk sac.

FIGURE 12. More advanced embryo 140 mm. in length. The dotted line at the proximal end of the umbilical cord (u.c.) shows the relative size of the yolk sac in a 220 mm. embryo.

#### ABBREVIATIONS

a. uterine artery, a.g. albumen gland, ap. apophyle, b.e. edge of blastoderm, c.d. closing device, cl. claw, clp. clasper, e.br.c. external branchial cleft, e.g. external gill filaments, em. 1, 2, 3. developing embryos, f.t. fallopian tube, f.t.o. coelomic orifice of same, g.r. gill rakers, hy. hypophyle, i.br.c. internal branchial cleft, l.ov. left ovary, m. mesentery, n.g. nidamental gland, ne.g. neural groove, ov. ovary, p. papillae, r. rhipidion, r.ov. right ovary, s. siphon, sp. spur, sp.g. spiracular gill filaments, spi. spiracle, u. uterus, u.c. umbilical cord, v. vagina, v.a. visceral arch, v.ar. vitelline artery, v.v. vitelline veins, ve. uterine vein, ven. venules, y.s. yolk sac.

maining yolk sac only 5 mm. in length. The embryos had the same coloration as the adults. It was not possible to determine the age of these embryos so that up to the present the gestation period is unknown.

## EXTERNAL GILLS

Gudger (1940, pp. 626, 627) writing about external gill filaments in shark embryos states—"The embryos of all nonplacental viviparous sharks and rays known to me have long external gills. The eggs of these Elasmobranchs have thin diaphanous shells, through which uterine fluids readily penetrate. These fluids are milk-like secretions of the uterine mucosa and serve as food for the growing embryos, which absorb this food through their long filamentous gills. It has been indicated that the relatively thick shells of *Chlamydoselachus* are burst by the growing embryo, are cast off into the uterus, and are then or later thrown out into the sea. Two investigators (Hawkes, 1907 and Smith, 1937) have found highly vascularized areas in the wall of the right uterus (cf. my Plate III, Fig. 10). These observations suggest that these areas might have served to secrete foodstuffs into the uterus. Then the long gestation period and the enormous size of the relatively late embryos still attached to large yolk sacs seem to indicate that these embryos grow not at the expense of the yolk alone. All these things lead to the inevitable question—"Do the external gills of the embryos of *Chlamydoselachus* serve to absorb food from a uterine secretion?" . . . It seems quite sure that in any case these external gills of the non-extruded juvenile sharks serve as respiratory organs."

Further, Gudger (op. cit., pp. 559-562) in dealing with the matter of intra-uterine gestation and respiration states (p. 561) "on the whole it seems quite probable that the young of both sharks (*Ginglymostoma* and *Chlamydoselachus*) may receive some oxygen by diffusion from the uterine wall into the fluids surrounding the embryo. Furthermore, from my knowledge of uterine gestation in other sharks and rays, I am strongly of the opinion that the uterine wall in both (sharks) secretes liquid food materials to nourish the young after they are freed from the egg capsules. . . . The long external gills of embryos of rays and of other sharks, when bathed in the uterine fluid, may take in not only oxygen but mineral salts and possibly other food substances as well. The rich plexus of vitelline capillaries will also be bathed in the fluid of the uterine cavity and they may absorb some food and oxygen from it. If this takes place in *Chlamydoselachus*, it must go on for a long time, until and even after the yolk is resorbed, and this yolk must be used up before the fish is born, else the free oceanic life of this little shark would be very brief."

There are many points of similarity between the present species and that described by Gudger. *Squalus acanthias* is ovoviviparous and at a certain stage of incubation the egg capsule bursts and the embryo is free to lie in the uterus. I have dissected gravid females in which parts of the capsule were found in the uterus. Plate IV, Figure 11, shows an embryo which lay completely free in the uterus with the remains of the capsule lying near the vagina, evidently ready to be extruded. The fact that it has been possible for me to keep some embryos of this species alive in sea water for some time after their removal from the uterus seems to indicate that the external gills must have a function purely respiratory. It is of interest to note that in all dissections of the uteri of gravid females the embryos lay with their heads pointed towards the cloaca and this leads me to infer that the relatively wide vaginal openings into the cloaca permit of sea water entering the uteri for the respiratory functions of the growing embryo. (Compare the provision of respiratory slits in the mermaid's purses of oviparous sharks through which water can enter to provide

respiration by means of the external gills—von Bonde, op. cit.) The external gill filaments are doubtless precocious growths of normal gills and they are eventually lost during later development. Compare Figures 11 and 12 which portray embryos of greatly varying stages of development. In the latter figure the external gill filaments have completely disappeared and the functional gills are typically adult in structure. The occurrence of external gill filaments protruding from the spiracular cleft is conclusive evidence that the spiracular cleft in sharks was primarily a gill cleft with a respiratory function.

Widakowich (1907a) states that the external gills are lost as soon as the egg capular membrane disappears. This is contrary to my observations on embryos kept alive artificially in sea water, and also on those which had burst the egg capsule and were lying free in the uterus, but he does corroborate the view that these transitory gills do not serve the purpose of taking up nutrient material.

There is an alternative possibility in connection with the respiration of the embryo during the time that it has the external gill filaments. In no case has it been possible to observe any connection between the mucous lining of the uterus and the yolk sac even faintly resembling a placenta. This occurrence of a placental development in *S. acanthias* is much featured by Widakowich (op. cit.), but it seems more probable that the papillae of the uterus, which are richly supplied with blood vessels, can, by a process of osmosis, aerate the blood of the developing embryo through the capillary system in the external gill filaments. As mentioned before, Ercolani (op. cit.) grouped this species under his second group of uterine developments and he mentioned the fact that these papillae are in close contact with the vitelline membrane of the yolk. I am convinced, however, that at no stage during the intra-uterine development of the embryo does the mother play any part in the nourishment of the embryo by means of the external gill filaments, the yolk sac alone functioning in this capacity. This contention is, moreover, strengthened by the fact that the yolk sacs in all gravid females dissected by me never showed any convolutions into which the vascular papilla of the uterus could fit. There is of course a possibility that here also there may be an osmotic transference of blood from the surface vascular papillae to the vitelline circulation, but I cannot see that such a function is necessary to the developing embryo. I, therefore, lean to the view that the sole function of these vascular papillae is to provide aeration through the external gill filaments, this function being in addition to that previously described where such aeration takes place by means of the sea water entering the uterus.

#### THE VITELLINE CIRCULATION

The earliest stages of the yolk sac circulation are shown in Plates II and III, Figures 7A and 7B. The vitelline blood vessels finally spread out over the whole yolk sac. Their function is to carry the nutrient yolk to the developing embryo. The early stages of the circulation were briefly described above. In all young embryos of *S. acanthias* a vitelline artery arises from the dorsal aorta and runs along the left side of the umbilical cord and thence forward from the yolk stalk (u.c.) over the surface of the yolk sac (v.ar.). In earlier embryos, this artery is sinuous (Plate II, v.ar.) but later it becomes straightened out. Its usual course runs from the umbilical cord to the anterior sharper apex of the yolk sac but there

are variations where it runs obliquely across the yolk sac to the sides. In every case, however, it curves over the yolk sac and branches into two, one arm being short whilst the other passes straight back along the ventral surface of the yolk sac (Fig. 7B, v.ar.) and then forks into two branches. On this ventral surface the vitelline artery gives off numerous side branches at right angles to itself whilst no branches are seen arising on the dorsal surface of the yolk. Posteriorly, on the dorsal surface there is a single main vitelline vein (Fig. 7A, v.v.) which drains a dendritic group of venules (ven.). This main vitelline vein runs along the right side of the umbilical cord and enters the heart with the splanchnic vein. In its course along the umbilical cord the main vitelline vein is joined by smaller veins near the yolk sac.

### SUMMARY

1. *Squalus acanthias* is an ovoviviparous dogfish with a wide range of distribution.
2. The male and female reproductive systems, especially the internal structure and the closing device of the uterus, are described.
3. Oviparous development takes place in a thin transparent egg capsule, different from the usual Selachian egg capsules, up to a certain stage, when the capsule ruptures and then the development becomes viviparous.
4. Embryos at different stages of development are described. Some capsules contained single embryos, others twins and still another triplets.
5. The functions of the external gills are dealt with, it being concluded that their function is primarily respiratory.
6. The vitelline circulation is described.

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## EMBRYONIC DEVELOPMENT IN POECILIID FISHES

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In *Heterandria formosa*, a viviparous cyprinodont fish of the family Poeciliidae, nearly all of the nourishment for embryonic development is obtained from the mother after fertilization through a pseudoplacental association (Scrimshaw, 1944). Similar although less complicated associations between mother and embryo exist in other species of this family (Turner, 1937, 1940). These species are for the most part considered to be ovoviviparous. The distinction is based on the difference between live bearing forms which retain an egg with a full supply of nourishment for development and those whose embryos receive nourishment from the mother. The latter are considered truly viviparous.

Turner (1937) referred to all species of Poeciliidae as ovoviviparous. He obviously used this term in a general sense for he suggested in discussing the data of Bailey (1933) that *Xiphophorus helleri* could receive nutriment from the parent. Turner also pointed out (1937) that the small *Heterandria* egg cannot contain enough nourishment to account for the size of the larvae and suggested that the follicle cells surrounding the embryo furnish food materials.

The weight values reported by Bailey for various embryonic stages in *Xiphophorus* show no decrease in the weight of the total yolk-embryo system. Since energy is used for maintenance metabolism, the total weight of this system decreases in forms depending entirely on yolk. Gray (1928) reported a decrease of 37 per cent for the oviparous trout, *Salmo fario*, and Hsiao (1941) found a decrease of 34 per cent in the truly ovoviviparous perch, *Sebastes marinus*. Accordingly, although he does not suggest this, Bailey's data show that some nourishment must be obtained from the mother by the developing embryo of *Xiphophorus*.

It is true that most poeciliid fishes are more dependent on the yolk laid down before fertilization than upon maternally supplied nourishment after that time. However, the evidence presented below shows that the members of this family do utilize nourishment outside of that contained in the yolk and hence are not ovoviviparous in the strict sense of the term. Embryos of such species as *Heterandria formosa* and *Aulophallus elongatus* are truly viviparous and as dependent on the mother for nourishment as are those of a placental mammal.

Gray (1926, 1928) reported the relationships between nourishment and growth rate in the oviparous trout, *Salmo fario*. The problems of oviparity and ovoviviparity are similar in that in each the embryo has its own supply of nourishment and receives food and water from its environment. When these conditions are compared with true viviparity, striking differences are noted (Scrimshaw, 1944). The present study was undertaken to find and describe intermediate stages between

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viviparity and ovoviviparity in other members of the family. Such stages would aid in the understanding of the complex problems of viviparity in this specialized group. The weight changes in the egg and embryos of twenty additional species of Poeciliidae have been determined and a condition intermediate between ovoviviparity and true viviparity characterized. There appeared a remarkable tendency for the young of all the species examined to receive from the mother only as much in terms of dry weight as they required for the maintenance of tissues already formed.

#### MATERIAL

Stocks of the following species were maintained in the laboratory and the gravid females were available as desired. These were dissected and the living embryos weighed.

|                                   |                            |
|-----------------------------------|----------------------------|
| <i>Gambusia affinis holbrooki</i> | <i>Quintana atrizona</i>   |
| <i>Lebistes reticulatus</i>       | <i>Xiphophorus helleri</i> |
| <i>Poeciliastes pleurospilus</i>  |                            |

The following species were collected in the field and the embryos weighed at the Institute for Research in Tropical America, Barro Colorado Island, Canal Zone (June–August, 1940).<sup>2</sup>

|                                    |                                |
|------------------------------------|--------------------------------|
| <i>Allogambusia tridentiger</i>    | <i>Brachyrhaphis episcopi</i>  |
| <i>Aulophallus elongatus</i>       | <i>Dariénichthes dariensis</i> |
| <i>Brachyrhaphis cascajalensis</i> | <i>Mollienisia sphenops</i>    |

In addition the writer is greatly indebted to Dr. Carl Hubbs of the Museum of Zoology, University of Michigan, for preserved material of the following species:

|                                  |                                      |
|----------------------------------|--------------------------------------|
| <i>Allophallus kidderi</i>       | <i>Poecilia vivipara</i>             |
| <i>Belonesox belizanus</i>       | <i>Poeciliopsis infans</i>           |
| <i>Brachyrhaphis terrabensis</i> | <i>Poeciliopsis occidentalis</i>     |
| <i>Dextripenis evides</i>        | <i>Poeciliastes pleurospilus</i>     |
| <i>Gambusia nobilis nobilis</i>  | <i>Pseudoxiphophorus bimaculatus</i> |
| <i>Mollienisia velifera</i>      | <i>taeniatus</i>                     |

Through the kindness of Dr. William Schroeder, additional specimens of *Gambusia affinis holbrooki* were obtained from the Museum of Comparative Zoology of Harvard University.

The gravid females of all of the above species were dissected and the contents of the ovary noted. In all of these forms the embryos are retained within the follicles of a large single median ovary which was removed intact before examination.<sup>3</sup> The embryos could be dissected out with the ovisac intact and the ap-

<sup>2</sup> This work was made possible by a grant from the Penrose Fund of the American Philosophical Society and the assistance of Dr. Thomas Barbour of the Museum of Comparative Zoology, Harvard University. The author was greatly assisted in the field work and weight measurements by Mr. Thomas C. Saunders of Harvard University. The cooperation of Mr. James Zetek, custodian of the island and laboratory; Mr. Graham Fairchild of the Gorgas Memorial Laboratory, Panama; and Mr. John Wood of the Canal Zone Police considerably aided this work.

<sup>3</sup> Dissection and weight data for all of these species are contained in the appendix of the author's doctorate thesis *Embryonic Growth in Poeciliid Fishes* deposited in the Harvard University Library.

proximate diameter measured with an ocular micrometer. The weight determinations were made as described for *Heterandria* (Scrimshaw, 1944), the embryos being dried on small cover slips over calcium chloride and weighed on a Sartorius balance. Any error in the actual dry weight recorded was insignificant in comparison with the variability of the material. Wet weights are not discussed because of their variability in preserved material and the difficulties in determining excess fluid. The taxonomic scheme followed throughout is that proposed by Hubbs (1924, 1926).

### EXPERIMENTAL

Dry weight determinations of the eggs and embryos in the intact ovisac were made at various stages between fertilization and parturition. In each case as many stages as could be obtained were studied and ranked according to age as described below for purposes of graphical and statistical representation.

It was soon observed that these weights were within the same general range regardless of the stage of the embryo. When least square lines were calculated through these points, a straight line parallel to the abscissa served to describe the data. Figures 1 and 2 show representative development lines and the scatter of weight values for four of these species.

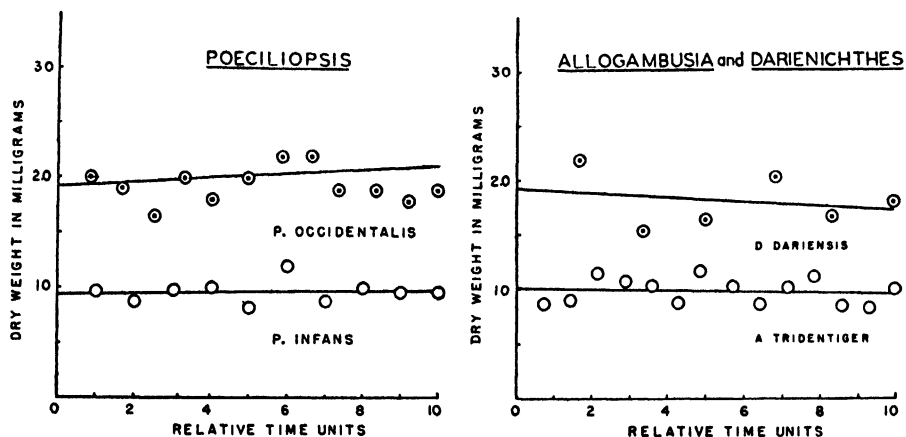


FIGURE 1. The dry weights of the embryos in the intact ovisac are arranged on a relative time axis in order of stage of development. For graphical and statistical purposes it has been necessary to assume that the time between stages is equal in all cases. Fertilization is represented at the beginning of the time scale and parturition at the end. The lines are calculated through these points by the method of least squares.

FIGURE 2. Two closely related species are portrayed in the same manner as those in Figure 1.

On the basis of these straight lines, the mean weight of the embryos of this group of poeciliid species can be compared (Figs. 3 and 4). The range is so great that all of the species could not be represented in one figure, but several forms are repeated in Figure 4 to facilitate comparisons. In addition to the species described

in this paper, *Heterandria formosa* and *Aulophallus elongatus*<sup>4</sup> are included as examples of viviparous poeciliid fish securing nearly all of their nourishment from the mother. The straight line for *Aulophallus* is purely diagrammatic, but its use

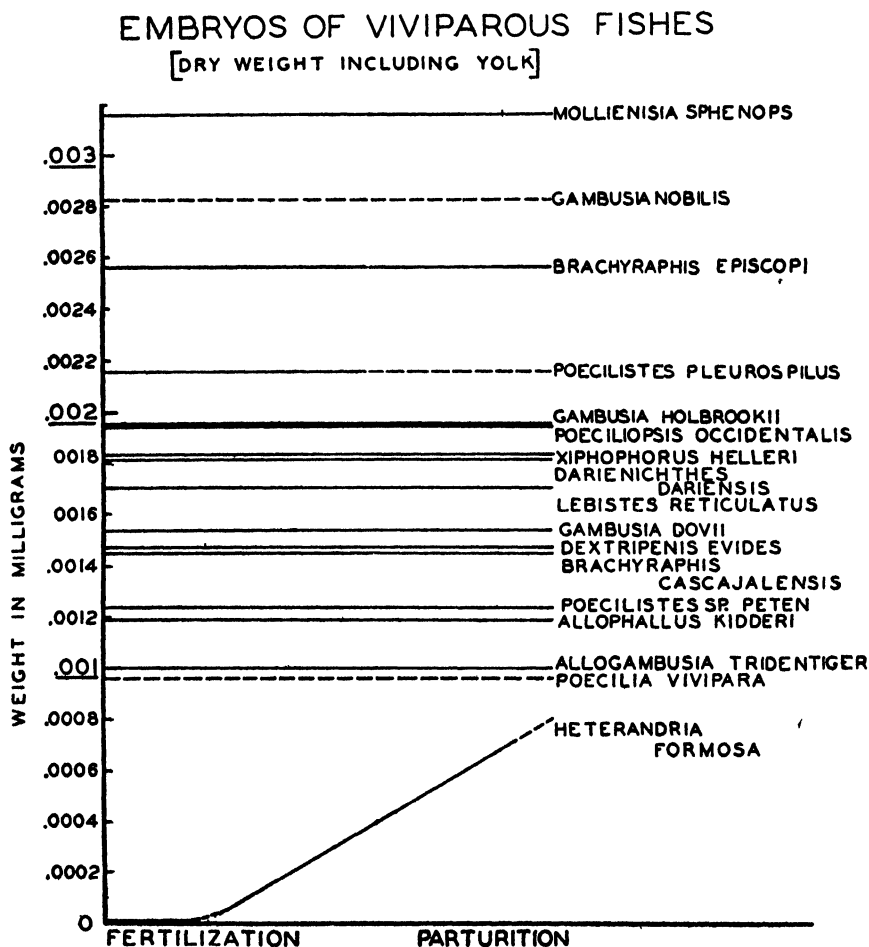


FIGURE 3. For purposes of comparison the dry weight curve of the embryo during development is drawn as a horizontal line. The average weight of all of the embryos of a given species is taken as the level at which the line is drawn. The degree to which these diagrammatic lines correspond to lines calculated through the data is discussed below. An approximation of the standard deviation observed from the above lines for each species is given by  $SE_y$  in Table I. *Heterandria formosa* is included as an example of a truly viviparous fish. The other species have previously been considered to be ovoviviparous. *Gambusia holbrooki* should be listed as *Gambusia affinis holbrooki*. *Poecilistes* sp. Peten has been identified by Hubbs as *Poecilistes pleurospilus* from Guatemala.

<sup>4</sup>Measurements on this species were also made in Panama during the summer of 1940. The weight of the fertilized egg and of the larva at parturition are indicated in Figure 1. A more complete discussion of this species is being prepared.

for *Heterandria* is probably legitimate (Scrimshaw, 1944). The broken lines indicate incomplete data. The known oviparous fishes have egg and larva weight greater than those of any of the fishes shown in Figure 1.

In Figures 1-6 no correction has been made for the effect of the fixative on the dry weight of the embryo. As can be seen from Figure 5, no single correction can

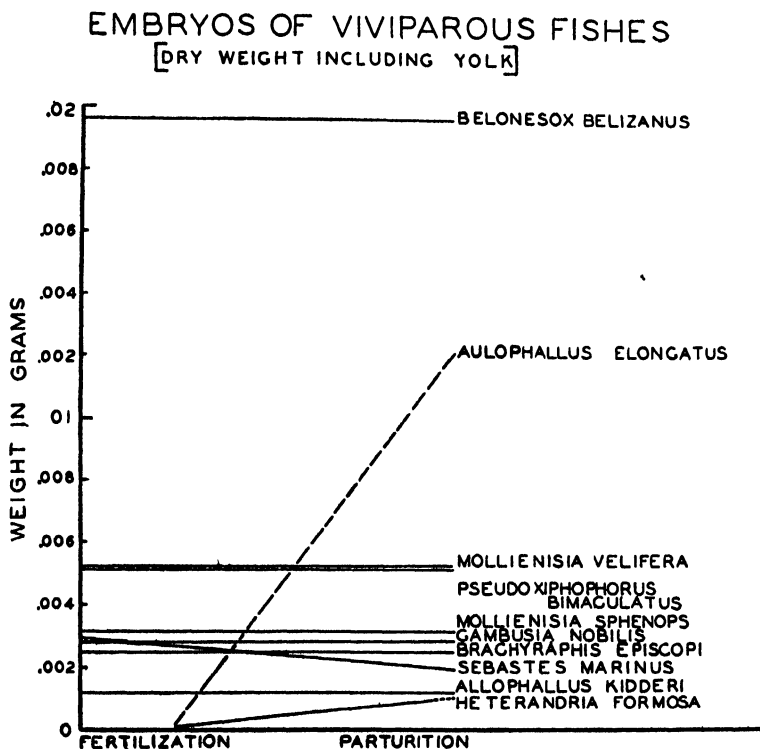


FIGURE 4. Additional species are shown in a manner identical to that described for Figure 3. The scale is enlarged to include species with greater embryonic weights. *Aulophallus* and *Heterandria* represent truly viviparous poeciliids. *Sebastes marinus* is a viviparous perch, a much larger fish than any of the others discussed and not a member of the Poeciliidae. It is included as an example of a truly ovoviviparous fish.

be made for preserved material. Some embryos of *Gambusia affinis holbrooki* preserved since 1878 have changed very little in dry weight when compared with living ones studied in the laboratory. On the other hand, certain material collected in 1919 from the same place as the laboratory stock collected in 1941 has lost at least 70 per cent of its original dry weight. These specimens, however, were fixed in alcohol and preserved in strong alcohol (as high as 90 per cent). The 1878 material was fixed in formalin before it was transferred to the strong alcohol used as a preservative for fish by the Museum of Comparative Zoology. The material obtained from the Michigan Museum of Zoology was uniformly treated, having been fixed in 10 per cent formalin and changed to 70 per cent alcohol for storage.

The dry weight of the embryo in fishes treated in this manner closely approximates that of the living form. The data plotted for *Brachyrhaphis cascajalensis* in Figure 2 illustrate this.

In Figure 4, the *Poecilistes* species has been identified by Hubbs as being a race of *Poecilistes pleurospilus* from Guatemala. The *Poecilistes pleurospilus* listed is laboratory stock obtained from the Everglades Aquatic Nurseries, Tampa, Florida. The marked difference in average weight of these two groups of specimens, assuming them to be the same species, is probably due to actual racial differences rather than to fixation. A similar situation was found for *Mollienisia*

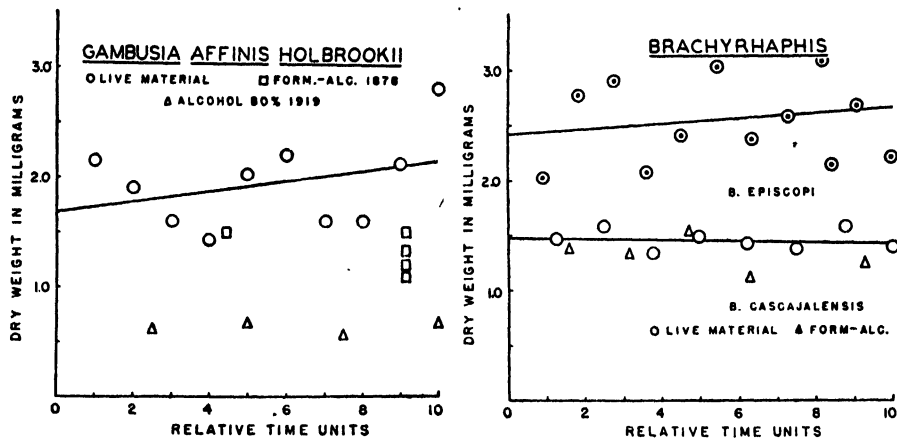


FIGURE 5. The dry weights of the embryo are plotted in a manner similar to Figure 1. The line is calculated from data represented by the circles. These represent embryos dissected out alive and dried immediately. The squares represent material preserved in formalin in 1878 and later transferred to the strong alcohol used in the Museum of Comparative Zoology, Cambridge, Mass. This varied between 75 and 90 per cent and was the sole preservative for specimens represented by the triangles.

FIGURE 6. The dry weights of the embryo are again plotted as in Figure 1. The triangles represent material preserved originally in 10 per cent formalin and later kept in 70 per cent alcohol. When weighed several months later there was excellent agreement with the values for living material studied at the time these were preserved. Most of the preserved material described has been handled in this manner.

*sphenops*. Two forms which could be distinguished by size, coloration, and mean weight of the embryos were taken from Gatun Lake. The mean weight of the larger form is plotted in Figure 3 as 3.05 mg. The smaller forms showed a very constant embryo weight averaging 1.58 mg. Despite these differences, Hubbs (1942) was able to find no usual taxonomic character or measurement to justify considering the smaller form a separate species.

Since the sperm in these fishes are viable in the ovary for several months, the time required to reach any given stage of development cannot be determined even in laboratory specimens. Therefore, in this study the embryonic weights were ranked in order of stage of development (cf. Scrimshaw, 1944). Since the data show that there is no consistent dry weight change during development, no error was introduced in the tabulation of the raw data. However, the assumption for

graphical and statistical purposes of an equal interval of time between the stages studied means the use of a relative rather than an actual time scale.

In the figures presented, the first stage was taken to represent the weight at fertilization and the last stage the weight at parturition. The time between was arbitrarily divided into ten units. Thus in the case of *Brachyrhaphis episcopi* where ten stages were studied, each stage in Figure 6 is separated by an arbitrary relative time unit of 1.0. In the case of *Allogambusia tridentiger* represented by fourteen stages (Fig. 2), the ten point scale must be divided into fourteen parts. Thus each stage is separated by 0.71 unit.

Using relative time units as the abscissa and dry weight in milligrams as the ordinate, the raw data were recorded graphically. It was apparent by inspection that the data for each species could be represented by a straight line. Accordingly these were fitted by the method of least squares and were of the form  $y = mt + k$ . The intercept ( $k$ ) of the straight line then represents the weight of the egg at fertilization. The slope ( $m$ ) describes the weight change during development.

TABLE I

| Species                               | N   | k             | SE <sub>y</sub> | m      | m°      |
|---------------------------------------|-----|---------------|-----------------|--------|---------|
| 1. <i>Gambusia affinis holbrooki</i>  | 10  | 1.676         | .1127           | .0502  | 2° 52'  |
| 2. <i>Darienchthys darsiensis</i>     | 6   | 1.925         | .0584           | -.0197 | -1° 8'  |
| 3. <i>Poeciliopsis occidentalis</i>   | 12  | 1.923         | .0456           | .0027  | 0° 9'   |
| 4. <i>Poeciliopsis infans</i>         | 10  | .937          | .0108           | .0046  | 0° 16'  |
| 5. <i>Aulophallus kidderi</i>         | 7   | 1.204         | .0167           | -.0040 | -0° 13' |
| 6. <i>Brachyrhaphis episcopi</i>      | 11  | 2.419         | .1237           | .0263  | 1° 30'  |
| 7. <i>Mollienisia sphenops</i>        | 12  | 3.045         | .1495           | .0204  | 1° 10'  |
| 8. <i>Dextripenis evides</i>          | 7   | 1.700         | .0266           | -.0350 | -2° 0'  |
| 9. <i>Brachyrhaphis cascajalensis</i> | 8   | 1.481         | .0076           | -.0055 | -0° 19' |
| 10. <i>Gambusia dovii</i>             | 10  | 1.667         | .0479           | -.0330 | -1° 53' |
| 11. <i>Allogambusia tridentiger</i>   | 14  | 1.005         | .0132           | -.0027 | -0° 9'  |
| 12. <i>Xiphophorus helleri</i> *      | 21  | 1.841         | .1602           | -.0009 | -0° 3'  |
| Total                                 | 118 | Average .0034 |                 |        | 0° 12'  |

N, number of embryonic stages; K, intercept of development curve in milligrams; SE<sub>y</sub>, standard error or estimate of y in milligrams,  $SE_y = \sqrt{\frac{\sum d_y^2}{N-1}}$ ; m, slope of development line; m°, deviation of development line from horizontal in degrees.

\* Calculated from data of Bailey (1933).

As listed in Table I, the mean slope of all of these lines is only .0034. This is equivalent to an angle 0° 12', an almost imperceptible deviation from a slope of 0. In order that these small deviations may be more readily visualized, the actual angle in degrees equivalent to the calculated slope is also listed in Table I. From inspection of the data, it can be concluded that the random variations from a slope of 0 can be accounted for by biological variation and sampling error. No significant change in the dry weight of the embryo in the intact ovisac can be detected during development.

A further statistical basis for the above conclusions can be obtained by placing the data for the different species in numerically comparable form.<sup>5</sup> This consists of adjusting the data already calculated to an arbitrary standard. The weight at fertilization ( $k$ ) was made identical for each species and equal to unity ( $k_r$ ). All other statistics were scaled accordingly by multiplication with the ratio  $1/k$ . The resulting curves ( $y_r = m_r t + k_r$ ) are seen in Figure 7 and the new relative statistics are listed in Table II.

TABLE II.

| Species                               | $m_r$  | $\hat{\sigma}m_r$ | $t_{0.00}$ | $t_{-.033}$ | $P_{0.00}$ | $P_{.033}$ |
|---------------------------------------|--------|-------------------|------------|-------------|------------|------------|
| 1. <i>Gambusia affinis holbrooki</i>  | .0300  | .0213             | 1.409      | 2.961       | .2         | .02        |
| 2. <i>Darienichthys dariensis</i>     | -.0102 | .0124             | -.8270     | 1.841       | .5         | .15        |
| 3. <i>Poeciliopsis occidentalis</i>   | .0014  | .0068             | .2032      | 5.028       | .5         | .001       |
| 4. <i>Poeciliopsis infans</i>         | .0049  | .0037             | .7434      | 7.464       | .5         | .001       |
| 5. <i>Aulophallus kidderi</i>         | -.0033 | .0053             | -.6324     | 5.653       | .5         | .001       |
| 6. <i>Brachyrhaphis episcopi</i>      | .0109  | .0154             | .7049      | 2.845       | .5         | .02        |
| 7. <i>Mollienisia sphenops</i>        | .0078  | .0142             | .5505      | 2.668       | .5         | .02        |
| 8. <i>Dextripennis evides</i>         | -.0206 | .0593             | -.3473     | .2093       | .5         | .5         |
| 9. <i>Brachyrhaphis cascajalensis</i> | -.0037 | .0018             | -2.044     | 6.088       | .09        | .001       |
| 10. <i>Gambusia dovii</i>             | -.0198 | .0091             | -2.183     | 1.452       | .05        | .2         |
| 11. <i>Allogambusia tridentiger</i>   | -.0027 | .0035             | -.7749     | 8.627       | .5         | .001       |
| 12. <i>Xiphophorus helleri</i> *      | -.0005 | .0190             | -.0268     | 1.709       | .5         | .1         |

$k_r$ , common reference intercept,  $k_r = 1.000$  (omitted from table, but essential part of data identical for all species);  $m_r$ , slope of relative development curve,  $m_r = m \cdot 1/k$ ;  $\hat{\sigma}m_r$ , unbiased estimate of standard deviation of  $m_r$ ;  $t_{0.00}$ , Fisher- $t$  statistic,  $t_{0.00} = \frac{m_r - 0.00}{\hat{\sigma}m_r}$ ;  $t_{.033}$ , Fisher- $t$  statistic,  $t_{.033} = \frac{m_r - (-.033)}{\hat{\sigma}m_r}$ ;  $P_{0.00}$ , probability that the difference in slope between the observed curve and one of zero slope is due to random sampling error;  $P_{.033}$ , probability that the difference in slope between the observed curve and one of  $-.033$  slope (parturition weight = 2/3 fertilization weight) is due to random sampling error.

With the statistical measures for the 11 species adjusted in this manner, the impressions gained from the raw data can be evaluated. It was pointed out above that these fishes have been considered by most authors to be ovoviviparous. As will be discussed, this would mean that one-third of the nourishment originally available in the egg would be used before parturition for maintenance purposes. If this is so, the slope of a straight line development curve would be  $-.033$  and the final dry weight would thus appear as  $\frac{2}{3}$  of the initial weight.<sup>6</sup>

The probability ( $P_{-.033}$ ) that the development curve of one of these species represents a chance deviation from a slope of  $-.033$  can be determined by using the relation  $t_0 = [m_r - (-0.033)/m_r]$  and tables of the Fisher- $t$  distribution. Examination of the  $P_{-.033}$  column in Table II shows that for most of the species examined the  $P$  is .05 or below. Thus, it is not probable that any of these curves represents a chance variation from a characteristic

<sup>5</sup> The statistical data in Table II have been calculated by Mr. James Rafferty of the School of Medicine and Dentistry, University of Rochester. The statistical treatment of the data has been carried out entirely by him and the text description of the methods employed has been prepared under his direction.

<sup>6</sup> The slope of such a line would be  $-.033$  if the units were equal for ordinate and abscissa. Since there are ten relative time units to a common reference weight of unity, the figure for the calculated slope becomes  $-.033$ .

slope of  $-0.033$ . To conclude that this group of curves as a whole shows chance deviation from such a slope is impossible. It should be apparent that the lines in Figure 7 do not represent a group of ovoviviparous species.

However, is the apparent grouping of the development curves about a slope of  $0.00$  significant? The probability ( $P_{0.00}$ ) that these lines represent chance variations from a characteristic slope of  $0.00$  can be found in a similar manner. It will be seen from Table II that the probability in most instances is  $.5$  or better that this is the case. Taken as a whole, it is probable that the modal development curve for this group of species is a horizontal line. Thus the conclusion that there is no change in the weight of the embryo in the intact ovisac is substantiated.

The data for certain of the species require further comment. The data for species 2, 10, and 12 do show  $P_{-0.003}$  values of  $.1 - .2$ . Of these, only species 10, *Gambusia dovii*, shows a real deviation from a line of  $0$  slope (Fig. 7). In the case of species 8, *Dextripenis evides*, with a  $P_{-0.003}$  value of  $.5$ , there is enough initial variation in the weights that a deviation from a slope of  $0.00$  or  $-0.033$  is equally probable.

It might also appear from Figure 7 that species 1, *Gambusia affinis holbrookii*, has pro-

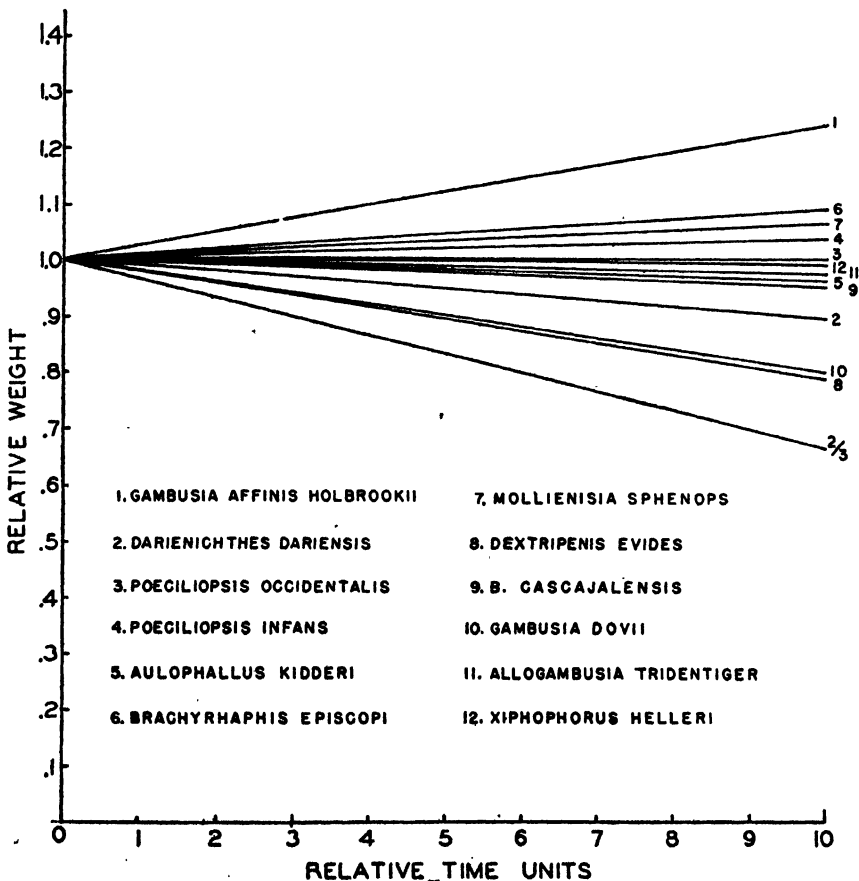


FIGURE 7. The weight data for the different species have been adjusted to an arbitrary standard so that the findings can be compared directly. The weight at fertilization ( $k$ ) was made identical for each species and equal to unity ( $k_r$ ). All other statistics were scaled accordingly by multiplication with the ratio  $1/k$ . Thus this new relative weight could be plotted as a function of relative time as used in the previous figures. The  $\frac{1}{2}$  line represents the line which the development curve of these species would follow if they were truly ovoviviparous.

gressed further than the other species toward true viviparity. While this possibility cannot be excluded, the  $P_0$  value of .20 may be taken to indicate that this deviation from zero slope may be a chance occurrence.

In several species it was not possible to secure enough stages to establish the mean dry weight of the embryo or the change in dry weight with development. The limited data for these species are summarized in Table III. The three females of *Lebistes* studied represented pure inbred laboratory stock and the average embryo weight of all three widely separated stages proved to be 2.3 milligrams. It is probable that most or all of the species in this table would fit into the group represented in Table I if complete data were available.

TABLE III  
Dry weight data for additional poeciliid species

| Species  | Number studied | Average weight (mg.) | $\sigma$ | Adequacy of data   |
|--|----------------|----------------------|----------|--|
| <i>Belonesox belizanus</i>                     | 6              | 19.7                 | —        | Range of 6.2–15.2 mg. for larva at parturition (6.2 value omitted from average)      |
| <i>Brachyraphis terrabensis</i>                | 5              | 2.18                 | .81      | Very narrow stage range and wide variation   |
| <i>Gambusia nobilis nobilis</i>                | 8              | 2.8                  | .80      | Only very early stages studied   |
| <i>Lebistes reticulatus</i>                    | 3              |                      | .0       | Too few stages   |
| <i>Mollienisia vivipara</i>                    | 3              | 5.4                  | —        | Few stages, very wide variation (3.4–6.3 mg.)  |
| <i>Poecilia vivipara</i>                       | 4              | .96                  | .67      | Too few stages   |
| <i>Pseudoxiphophorus bimaculatus taeniatus</i> | 5              | 5.3                  | —        | Early stages only (Excessively low late stage value of 3.2 mg. omitted from average) |

## DISCUSSION

The variation in the weight of the intact ovisac at various embryonic stages seems to be a characteristic of the particular population of a species sampled. As described above, the standard error of  $y$  ( $SE_y$ ) is actually a composite of errors. Nevertheless, it is primarily a reflection of the physiological and genetic variability to be found within the representatives of the species studied. It is probably valid to compare the inherent genetic variability of one species sample with that of another by means of this statistic. When the  $SE_y$  is small enough to minimize overlapping, the weight of the embryos should occasionally prove of taxonomic use.

The experimental data show the weight of the embryo at parturition to be the same as that of the egg at fertilization. Thus, quantitatively at least, the full amount of food material in the egg is converted into the substance of the embryo at the time of hatching. However, it is known that the food requirement of the embryo for maintenance metabolism during development is considerable.

Gray (1928) obtained for the plastic efficiency coefficient (P.E.C.) of the trout a figure of 0.63. This he believes to be a universal value for the synthesis of

protoplasm. This coefficient is based on the dry weight of the yolk that can be accounted for by the dry weight of the fully developed larva. It represents the relative cost in weight units of building the tissues of the embryo. The higher the coefficient, the smaller the amount of burned substance in relation to the amount of nourishment initially available. Table IV summarizes the values for P.E.C. which have been reported. These values suggest that the mother must also contribute an amount of nourishment equal to about one-third the initial weight in the poeciliid fishes studied.

TABLE IV  
*Plastic efficiency coefficients of development*

| Species                                      | Author                         | Coefficient             |
|--|--------------------------------|-------------------------|
| Trout ( <i>Salmo fario</i> )                 | Gray (1926)                    | .63 embryo              |
| Chick ( <i>Gallus domesticus</i> )           | Murray (1926)                  | .68 embryo              |
| Frog ( <i>Rana temporaria</i> )              | Fauré-Fremiet & Dragoiu (1923) | .58 embryo              |
| Silkworm ( <i>Bombyx mori</i> )              | Farkas (1903)                  | .59 embryo              |
| Mould ( <i>Aspergillus niger</i> )           | Terroine & Wurmser (1922)      | .59 normal growth       |
| Viviparous perch ( <i>Sebastes marinus</i> ) | Hsiao (unpublished data)       | .66 embryo              |
| Trout ( <i>Savelinus fontinalis</i> )        | Gortner (1912)                 | .74 embryo (heat dried) |

The maintenance cost of development is also illustrated by the various measures of the energy required during development. This subject has been well summarized by Needham (1931). Table V shows the values which have been obtained experimentally for one type of energy coefficient, the apparent energetic

TABLE V  
*Apparent energetic efficiency of embryonic development*

| Species  | Author                              | Efficiency |
|--|-------------------------------------|------------|
| Chick ( <i>Gallus domesticus</i> )             | Tangl (1903)                        | 62.9%      |
| Chick ( <i>Gallus domesticus</i> )             | Murray (1926)                       | 67.0       |
| Fundulus ( <i>Fundulus heterolitus</i> )       | Glaser (1912)                       | 52.8*      |
| Frog to hatching ( <i>Rana temporaria</i> )    | Fauré-Fremiet & V. du Streel (1921) | 82.0       |
| Frog to end of yolk ( <i>Rana temporaria</i> ) | Fauré-Fremiet & Dragoiu (1923)      | 51.0       |

\* This value was obtained by assuming that the same energy relationships prevailed for the use of the final half of the yolk as for the initial half. This figure is certainly too low.

efficiency (A.E.E.), which gives the relation between the chemical energy in the fertilized egg and that combusted during development. For example, the figure cited by Tangl for the chick would indicate simply that 37.1 per cent of the original energy stored in the egg was lost by the time the yolk was consumed and the embryo well formed. All of the available evidence indicates that it is legitimate to generalize and to conclude that in the course of embryonic growth of animals developing from a yolked egg, about one-third of the energy initially available is expended for maintenance metabolism.

The evidence that this relationship is also true for viviparous animals is more difficult to obtain. Rubner (1908) derived values, in part theoretical, for the effi-

ciency of seven common mammals (horse, cow, sheep, pig, dog, cat and rabbit). The agreement of these values with the above data is sufficiently close to permit Needham (1931) to conclude that the real energetic efficiency (R.E.E.) of all embryos, mammalian as well as non-mammalian, is about .66. The R.E.E. involves a correction for basal metabolism, but for poikilothermic animals (and embryos such as those of the chick which are essentially cold blooded for the greater part of their prenatal life) it is so close to the A.E.E. that the two can be considered together.

An energy loss of about 34 per cent in the poeciliid fishes studied should be reflected in an appreciable decrease in the total amount of organic matter. The fact that no change in dry weight can be detected points to an exact correspondence between the weight of food used for maintenance and that of the nourishment supplied by the mother. But why should the majority of species in the family Poeciliidae stop at this identical point in the development of viviparity? Some similar factor or factors must be limiting the growth of the embryo to the initial weight of the egg in all of these species.

This might be the case if some essential factor in the yolk were present in a limiting quantity. If this factor could not be supplied by the mother, no amount of energy from her would permit the embryo to grow larger than the initial supply of this limiting substance would allow. Such might be the case if, for example, only simple sugars and certain amino acids were able to cross the placental barrier. Then certain of the essential amino acids, fats, vitamins or other factors might be available to the embryo in restricted quantities. However, the exact correspondence between initial and final weight in nearly all the species is difficult to understand on this basis.

In these fish the nourishment must pass through five to seven tissue layers: the maternal endothelium, maternal connective tissue (theca), maternal epithelium (follicular epithelium), possibly the chorion and vitelline membrane, yolk sac epithelium, embryonic endothelium, and probably some embryonic connective tissue. This situation resembles most closely the epitheliochorial type of placenta found in such animals as the pig. Despite the number of membranes in this type of placenta all of the materials necessary for growth and maintenance pass from the mother to the fetus across this barrier.

In some mammals, the materials most important for the elaboration of embryonic tissues, the amino acids, pass through the placenta against a concentration gradient in a manner suggesting a special mechanism (Needham, 1942). If a completely viviparous fish such as *Heterandria formosa* possessed such a mechanism, the steady gain in weight of its embryos can be understood. If, on the other hand, the fish discussed here lack such a mechanism, the failure of their embryos to gain weight might also be accounted for.

It is also of interest to note that carbohydrate is absent from eggs of oviparous fishes. Needham (1931), in summarizing the available data, expresses the belief that this is true because of the impossibility of supplying an oviparous form with sufficient sugar for embryonic metabolism without vastly increasing the size of the egg. However, there is no reason to believe that carbohydrate would not be used by their embryos in preference to the stores of fat and protein, if it were available. In the case of the mammalian fetus, Windle (1940, page 212) concludes from the available evidence that the fetus of the mammal practices a rigid glycogen

economy at the expense of its mother's dextrose, drawing upon its own liver only in emergencies. It seems reasonable to expect that carbohydrate would also be the most readily available food which the embryo of the viviparous fish can obtain from the mother. Perhaps in the species discussed, carbohydrate from the mother exerts some sparing action on the tissue building materials of the yolk.

The discussion has been carried to this point to suggest something of the direct study which should be made on the functioning of the pseudo-placental barrier in these forms. The controlling mechanisms which are actually operating in the development of the species reported will not be clear until such experiments are carried out.

#### SUMMARY

In poeciliid fishes the embryos are retained within the ovarian follicles until parturition. When the dry weight of the intact follicle is determined at various stages of development, no significant weight change can be demonstrated in the 18 species studied. This is in marked contrast to the situation in a truly ovoviviparous fish where one-third of the initial weight of the egg is used for maintenance during development. Accordingly, it is concluded that all of the species studied receive nourishment from the mother and hence are not ovoviviparous in the strict sense of the word. Furthermore, they apparently receive only as much nutriment, quantitatively at least, as they require for their maintenance metabolism. Some limiting mechanism must be operating, but its nature is not known. This situation is contrasted with that in *Heterandria formosa* and *Aulophallus elongatus*, members of the family which have developed nearly complete dependence on the mother for nourishment during development.

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# THE EFFECT OF TEMPERATURE ON THE WINGS OF DIMORPHOS/DIMORPHOS VESTIGIAL-PENNANT/ VESTIGIAL IN DROSOPHILA MELANOGASTER

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Harnly (1930b) and Stanley (1931) showed a critical temperature for the increase in wing size of the homozygous vestigial flies. This response is direct but not proportional to the rise in temperature. It was found that the introduction of the sex-linked modifier dimorphos into this genotype lowered the critical temperature for wing enlargement 5° C. for the males and 2° C. for the females (Harnly and Harnly, 1935). Again the response was direct but not proportional throughout the viable temperature range. Vestigial-pennant is a reverse mutation from vestigial to a recessive wild type allele. The wings of these flies showed an inverse response to temperature, and a critical point appeared to occur between 28° and 30° (Harnly and Harnly, 1936). It was also found that the heterozygous vestigial-pennant/vestigial wings showed an inverse response to temperature from 16° to 22° and a direct response from 26° through 32°.

These shifts in critical temperatures, the direct or inverse response of wing size to temperature of the homozygotes, and the apparent reversal of dominance with temperature in the heterozygote led to this study of the dimorphos/dimorphos vestigial-pennant/vestigial genotype.

## STOCKS AND METHODS

Through a knowledge of the phenotypes produced by dimorphos vestigial, homozygous vestigial-pennant and the heterozygous vestigial/vestigial-pennant at 22° and 25°, it was possible to develop a stock of dimorphos vestigial-pennant. Iso-genic stocks of dimorphos vestigial and dimorphos vestigial-pennant were employed in these experiments. Due to the breeding technique (Harnly and Harnly, 1935, 1936) these stocks differ from each other in genotype only at the vestigial locus.

The culture methods, procedure for measurement of wings, etc. were the same as those described in earlier papers of this series (Harnly, 1932, 1936; Harnly and Harnly, 1935). Homozygous dimorphos vestigial-pennant females were mated to homozygous dimorphos vestigial males. Their offspring (dimorphos/dimorphos vestigial-pennant/vestigial) were allowed complete development at 16°, 18°, 20°, 22°, 24°, 26°, 28°, and 30° C. Those F<sub>1</sub> individuals placed at 32° for development were returned to a 26° incubator after the completion of five days (120 hours) of development and there completed their growth and their emergence.

## RESULTS

### *Wing length*

The genes used and their symbols are: vestigial (vg), vestigial-pennant (vg<sup>p</sup>) and dimorphos (di). The curves for the mean wing length for the di/di vg<sup>p</sup>/vg

males and females show an inverse response to temperature increases from 16° to 28°, and are similar in slopes and values to those previously recorded for the wild type (Stanley, 1935) and homozygous  $vg^p$  (Harnly and Harnly, 1936). The mean wing lengths in mm. of  $di/di\ vg^p/vg$  males and females are shown in Table I.

TABLE I  
*Mean wing length in mm.,  $di/di\ vg^p/vg$ , 5 trials*

| ° C. | Males  |   |        |          | Females |   |        |          |
|------|--------|---|--------|----------|---------|---|--------|----------|
|      | Length | ± | P.E.   | $\sigma$ | Length  | ± | P.E.   | $\sigma$ |
| 16°  | 2.53   | ± | 0.0097 | 0.1241   | 2.60    | ± | 0.0089 | 0.1135   |
| 18°  | 2.49   | ± | 0.0056 | 0.0724   | 2.59    | ± | 0.0103 | 0.1306   |
| 20°  | 2.42   | ± | 0.0055 | 0.0707   | 2.53    | ± | 0.0086 | 0.1101   |
| 22°  | 2.32   | ± | 0.0082 | 0.1030   | 2.43    | ± | 0.0119 | 0.1524   |
| 24°  | 2.25   | ± | 0.0069 | 0.0897   | 2.33    | ± | 0.0121 | 0.1550   |
| 26°  | 2.25   | ± | 0.0066 | 0.0755   | 2.35    | ± | 0.0109 | 0.1257   |
| 28°  | 2.11   | ± | 0.0073 | 0.0939   | 2.27    | ± | 0.0094 | 0.1205   |
| 30°  | 2.15   | ± | 0.0067 | 0.0859   | 2.28    | ± | 0.0067 | 0.0864   |
| 32°  | 2.24   | ± | 0.0081 | 0.0969   | 2.32    | ± | 0.0069 | 0.0886   |

The  $di/di\ vg^p/vg$  males and females show a gradual decrease in wing length from 16° to 28° beyond which there is a gradual increase in wing length to 32°. Apparently 28° is a critical point since the slope is negative below this temperature and is positive above it. The female wings are consistently longer than those of the males throughout the temperature range. The similarity in slopes and values of the  $di/di\ vg^p/vg$  male and female curves indicates there was no difference in the response of the two sexes in wing length over the range employed.

#### *Wing area*

The mean wing areas for  $di/di\ vg^p/vg$  will be found in Table II and are shown

TABLE II  
*Mean wing area in sq. mm.,  $di/di\ vg^p/vg$ , 5 trials*

| ° C. | Males |   |        |          | Females |   |        |          |
|------|-------|---|--------|----------|---------|---|--------|----------|
|      | Area  | ± | P.E.   | $\sigma$ | Area    | ± | P.E.   | $\sigma$ |
| 16°  | 1.591 | ± | 0.0090 | 0.1451   | 1.327   | ± | 0.0171 | 0.2161   |
| 18°  | 1.555 | ± | 0.0071 | 0.0914   | 1.171   | ± | 0.0171 | 0.2168   |
| 20°  | 1.482 | ± | 0.0086 | 0.1106   | 1.065   | ± | 0.0151 | 0.1934   |
| 22°  | 1.338 | ± | 0.0086 | 0.1080   | 0.964   | ± | 0.0156 | 0.2006   |
| 24°  | 1.278 | ± | 0.0219 | 0.2313   | 1.063   | ± | 0.0200 | 0.2568   |
| 26°  | 1.267 | ± | 0.0064 | 0.0730   | 1.111   | ± | 0.0163 | 0.1867   |
| 28°  | 1.138 | ± | 0.0080 | 0.1026   | 1.219   | ± | 0.0116 | 0.1492   |
| 30°  | 1.214 | ± | 0.0062 | 0.0796   | 1.294   | ± | 0.0065 | 0.0840   |
| 32°  | 1.291 | ± | 0.0080 | 0.0957   | 1.342   | ± | 0.0093 | 0.1192   |

in Figures 1 and 2 together with those of Harnly (1930b) and Harnly and Harnly (1935, 1936) for the genotypes indicated.

The male curves for  $vg^P$  and  $di/di\ vg^P/vg$  coincide and have a negative slope between  $16^\circ$  and  $28^\circ$ . Between  $28^\circ$  and  $32^\circ$  they diverge sharply, the homozygote having a higher negative value while the heterozygote has a definitely positive slope. Apparently  $28^\circ$  is the critical point for length and area in the males of both genotypes.

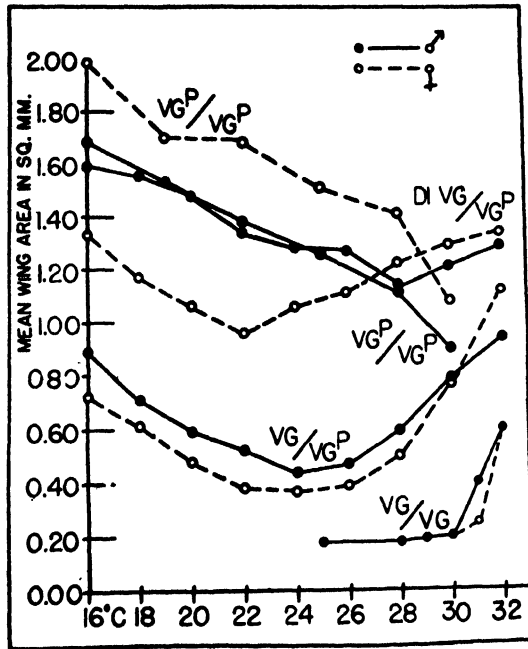


FIGURE 1.

The curve for the  $di/di\ vg^P/vg$  female wing area is U-shaped as is that of  $vg^P/vg$  females between  $16^\circ$  and  $32^\circ$ , but their wings are approximately double the area of the  $vg^P/vg$  wings. From  $16^\circ$  to  $22^\circ$  there is a gradual decrease and from  $22^\circ$  to  $32^\circ$  there is a steady increase in the area of the  $di/di\ vg^P/vg$  female wings. The reversal in the slope of the curve at  $22^\circ$  demonstrates that this is the critical temperature for the  $di/di\ vg^P/vg$  females. Between  $26^\circ$  and  $28^\circ$  the female curve crosses the male curve indicating a larger wing area for the females beyond that point up to  $32^\circ$ .

#### SHIFT IN WING FORM THRESHOLD

The male wing phenotype did not vary significantly from  $16^\circ$  to  $32^\circ$  in the  $di/di\ vg^P/vg$  flies. However, this genotype appears to be responsive to temperature, in that the percentage of perfect wings varied with the temperature. Wings with minor nicks were produced at all temperatures, but perfect wings were only

produced at 16° (19%), 18° (11%), 26° (10%), 28° (7%), 30° (7%), and 32° (28%).

The original homozygous  $vg^p$  stock showed only occasional nicks in the distal margins of the wings (Harnly and Harnly, 1936). Repeated back crossing of the  $vg^p$  to the inbred  $vg$  stock has introduced one or more wing margin genes intensifying this tendency. This resulted in a  $vg^p$  stock that regularly showed minor nicks at all temperatures, but produced perfect wings at 16° (Harnly and Harnly, 1936). Due to their origin the homozygous  $di\ vg^p$  and the homozygous  $di\ vg$  stocks presumably carried the same wing margin gene or genes for the production of these minor nicks or notches.

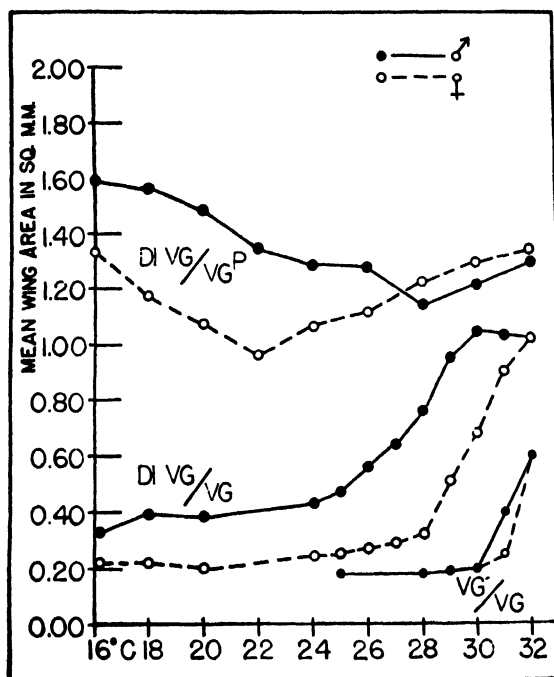


FIGURE 2.

The variation in frequency of nicks through the visible temperature range indicates that the wing-margin gene or genes are thermolabile at some period during the development of the individual. The largest number of perfectly normal wings appear at the temperature extremes, namely 16° and 32°. The smaller percentages of normal wings are found at temperatures close to the upper extreme. This indicates that some genes affecting wing margins are expressed at low temperatures while others find expression at high temperatures. Harnly (1942) obtained parallel results from reciprocal crosses between the two  $vg^p$  stocks and between these and a new  $vg^p$  stock. The  $di/di\ vg^p/vg$  genotype evidently produces full wings in the males with the possibility of minor variations at the distal end through the action of these wing-margin genes.

The  $di/di\ vg^p/vg$  female wings offer an entirely different picture. From  $16^\circ$  through  $26^\circ$  the phenotype varied around over-sized "antlered" with the occasional appearance of a "strap" wing. At  $24^\circ$  and at  $26^\circ$  phenotypes resembling the vestigial alleles antlered, snipped, carved and notched also appeared; from  $28^\circ$  to  $32^\circ$  the wings were predominantly "notched" to "nicked." Perfect normal wings appear only at  $32^\circ$  and 24 per cent of these wings were normal.

The  $di/di\ vg^p/vg$  female wings at  $16^\circ$  were similar to the male wings of the  $vg^p/vg$  described by Harnly and Harnly (1936) at  $16^\circ$ . The introduction of  $di$  into the  $vg^p/vg$  genotype has lowered the threshold for the wing pattern sequence previously described (Harnly and Harnly, 1935). This results in a pattern grade (antlered) for the female at  $16^\circ$  the equivalent of that first observed between  $28^\circ$

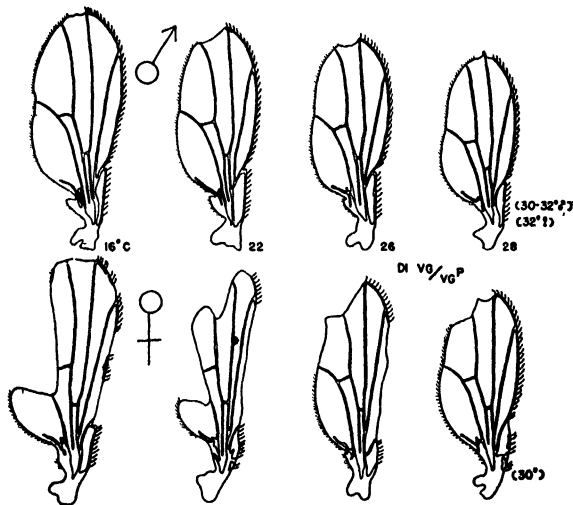


FIGURE 3.

and  $30^\circ$  in the genotype (female  $vg^p/vg$ ) carrying the normal allele of this sex-linked modifier (Harnly and Harnly, 1936). At these higher temperatures the  $di/di\ vg^p/vg$  females graded phenotypically "notched" to "nicked." The  $di$  gene had lowered the pattern threshold some  $12^\circ$  in the females and had lowered it so far in the males that only the full-wing pattern grade was produced throughout the viable temperature range.

#### SHIFTS IN THE CRITICAL TEMPERATURE

The  $di$  mutation arose in an inbred homozygous  $vg$  stock and consequently the  $di\ vg$  genotype differed from this inbred  $vg$  strain only in the allele present at the  $di$  locus. The original  $vg^p$  mutant arose in a culture of black vestigial flies. This mutant had been backcrossed for many generations to the inbred  $vg$  stock. This produced a  $vg^p$  line differing from the inbred  $vg$  strain by only the allele at the  $vg$  locus. Therefore, these three genotypes differed only by known alleles at the  $vg$  and  $di$  loci. Consequently the introduction of  $di$  into this backcrossed  $vg^p$  stock

produced a change in only one locus. The experiments on  $vg$ ,  $di\ vg$ ,  $vg^p/vg$  and  $vg^p$  (unpublished) have been reported previously (Harnly, 1930, 1936; Harnly and Harnly, 1935, 1936). Including our own experiments on  $di/di\ vg^p/vg$  truly comparable data is now available on five isogenic genotypes.

The  $di/di\ vg^p/vg$  male wings are phenotypically wild type throughout the temperature range employed. However, as mentioned above, "nicks" occasionally appear at the distal margins of the wings. These "nicks" are due to wing-margin genes (Harnly, 1942). The curves for male wing length and wing area coincide with the wild and  $vg^p$  curves and demonstrate a similar response to temperature between  $16^\circ$  and  $28^\circ$ . The reversal in the sign of the slope of the  $di/di\ vg^p/vg$  curves between  $28^\circ$  and  $32^\circ$  is markedly contrasted with the further fall of the wild and  $vg^p$  curves. This response is due to the combined action of the  $di$  and  $vg$  genes as will be shown later. The female length curve of  $di/di\ vg^p/vg$  parallels that of the males throughout the temperature range. Evidently under the conditions in these experiments, the threshold for the wild phenotype and temperature reactions of the male and the length response for the female has been reached in this genotype.

The critical temperature is a function of the genotype. The response of  $vg$  is direct with temperature and the value of the slope of the curve increases sharply above  $30^\circ$ . The  $di$  gene lowers this critical temperature  $2^\circ$  in the homozygous  $vg$  female. The slope of the wing area curve of homozygous  $vg^p$  females is negative in sign. The wing area curve of the heterozygote  $vg^p/vg$  has a negative slope from  $16^\circ$  to  $24^\circ$  and a positive slope from that point to  $32^\circ$ . The area curve of the  $di/di\ vg^p/vg$  females has a negative slope from  $16^\circ$  to  $22^\circ$ , and a positive slope from there to  $32^\circ$ . Again, the introduction of the dimorphous gene has lowered the critical temperature  $2^\circ$ , in the females.

These shifts in the critical temperature from genotype to genotype may be explained as the result of changes in dominance. On that basis the critical temperature (e.g.  $24^\circ$  for  $vg^p/vg$ ) is the point at which a reversal in dominance occurs between the alleles  $vg^p$  and  $vg$  in the heterozygote as was suggested earlier by Harnly and Harnly (1936). The data from these experiments with  $di/di\ vg^p/vg$  indicate that there may be two critical temperatures: (1) the reversal of dominance as indicated in the wing area curves; and (2) the phenotype threshold (discussed above), a problem of differentiation as opposed to that of growth in development. It may be that further work will show that both of these are different manifestations of the same phenomenon.

#### SUMMARY

1. The genotype  $di/di\ vg^p/vg$  was examined at  $2^\circ$  intervals from  $16^\circ$  to  $32^\circ$ .
2. The length of the wings on the males and the females decreased from  $16^\circ$  to  $28^\circ$  and increased from  $28^\circ$  to  $32^\circ$ . The wings of the females were longer than those of the males at all temperatures.
3. The area of the wings of the males decreased from  $16^\circ$  to  $28^\circ$  and then increased to  $32^\circ$ .
4. The critical temperature for both the length and area of the male wings is apparently  $28^\circ$ .
5. The area of the wings of the females decreased from  $16^\circ$  to  $22^\circ$  and increased from  $22^\circ$  to  $32^\circ$ . The resulting U-shaped curve is very similar to that reported

previously for  $vg^p/vg$  but the values are approximately double for the  $di/di\ vg^p/vg$  genotype.

6. The critical temperature for the female wing area appears to be at  $22^\circ$  and for wing length at  $28^\circ$ .

7. The  $di$  gene lowers the critical temperature for wing area  $2^\circ$  in the  $vg/vg$  female and the  $vg^p/vg$  female.

8. The critical temperature is a function of the genotype.

9. The males have wild type wings with minor marginal nicks at all temperatures. Wings with perfect margins were produced at  $16^\circ$  (19%),  $18^\circ$  (11%),  $26^\circ$  (10%),  $28^\circ$  (7%),  $30^\circ$  (7%), and  $32^\circ$  (28%). The marginal defects are obviously thermolabile.

10. The wings of the females varied around over-sized "antlered" with the occasional appearance of a "strap" wing from  $16^\circ$  to  $26^\circ$ . Phenotypes resembling the mutant alleles antlered, snipped, carved, and notched appeared at  $24^\circ$  and  $26^\circ$ . The wings were predominantly "notched" to "nicked" from  $28^\circ$  to  $32^\circ$ . Perfect margins were observed on twenty-four per cent of the wings developed at  $32^\circ$ .

11. The  $di$  gene lowered the wing pattern threshold some  $12^\circ$  in the females. In the males, this threshold was lowered below the viable range and only normal wings were produced.

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# SUBSTRATE-ENZYME ORIENTATION DURING EMBRYONIC DEVELOPMENT<sup>1</sup>

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## INTRODUCTION

Since Godlewski's (1900) observations it has been known that the rate of respiration increases during the course of amphibian development. Numerous workers (cf. Moog, 1944) have confirmed these results with more accurate measurements. The work of Loeb (1895) and Philips (1940) on *Fundulus* embryos and Brachet (1934) on amphibians made it evident that a rigid causal dependence of embryogenesis on oxygen consumption does not obtain in all instances. These authors found that a considerable portion of early embryonic development could proceed in the complete absence of oxygen.

Relatively few attempts have been made to analyze experimentally the increase in oxygen consumption during development. Such an increase could presumably involve: (1) an increase in the permeability of the eggs to oxygen during development, (2) synthesis or activation of more enzymes, (3) formation of additional substrate, and (4) reorientation of enzyme and substrate, initially present, but functionally disconnected. This could be accomplished by a spatial separation between the two and the immobilization of a necessary carrier.

The first mechanism is made unpalatable by the findings of Parnas and Krasinska (1921), confirmed by Brachet (1934), who found the respiratory rate of amphibian embryos to be, within wide limits, independent of oxygen tension up to neurulation. During this same period a 4 to 5 fold increase in rate of oxygen consumption is realized.

It seemed probable that a systematic comparison of the respiration of brei and intact eggs during development combined with an examination of the cytochrome oxidase content could provide data which would permit a decision amongst the last three hypotheses mentioned. The present paper presents the results obtained from such experiments. They support the hypothesis that control of respiratory rate in developing embryos is effected by a spatial orientation of enzyme and substrate. No detectable increase in the cytochrome oxidase content was observed and the substrate content decreases rather than increases during development.

## MATERIALS AND METHODS

### *Eggs*

The eggs of *Rana pipiens* were obtained after injection of anterior pituitary gland. They were artificially fertilized and after swelling of the jelly were cut up

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into small groups in 10 per cent Ringers solution and kept at 20° C. The stages at which the eggs were selected for measurements were determined according to the schedules of Pollister and Moore (1937) and Shumway (1941). Where desirable intermediate stages were devised with the aid of photographs published in Rugh's (1941) manual.

In preparation for an experiment, the eggs were plucked clean of their jelly with the aid of a pair of fine forceps and filter paper. The denuded eggs were immediately placed in the M/15 phosphate buffer at pH 6.5. The number of eggs used in each flask varied at different stages. The attempt was made to use enough material to give ca. 5 mm. movement of the manometer fluid for each ten minute reading. The eggs were suspended in sufficient fluid so that the total volume was 2 cc. and transferred to the respirometer vessel.

#### *Manometric measurements*

All measurements were carried out at 26.2° C. in Barcroft-Warburg Manometric respirometers. The vessels were provided with side arms for substrate additions. The rate of shaking was 100 complete oscillations per minute with an amplitude of 8 cm.

#### *Preparation of brei*

After some preliminary experiments the following was adopted as the most satisfactory available method for obtaining breis of the eggs: The correct number of eggs was diluted to 20 cc. with chilled M/15 phosphate buffer and the suspension placed in a Waring blender. This volume is sufficient to almost cover the blades. When for various reasons less volume was used, tilting of the blender was resorted to during its operation. The blender was turned on intermittently until complete destruction of cell structure was attained as determined by direct microscopic observations. Care was taken to keep the temperature of the fluid below 25° C. during the process. Aliquots of the cell-free suspension were then dispensed to the vessels.

It may be noted that attempts to prepare cell-free breis by grinding with purified sand invariably led to considerable inactivation of the respiratory enzymes as evidenced by the consistently lower rates of oxygen consumption of such preparations as compared with those made with the blender. We had no success with the violent shaking procedure described by Brachet (1934). Fewer than 20 per cent of the embryos were disrupted by this method which in Brachet's hands yielded almost 100 per cent cytolysis. This difference may be attributed to a relatively greater sensitivity of the European species (*Rana temporaria*) to mechanical disruption.

The respiration of the breis starts to decline after about 60 minutes. Consequently rates were determined in the earlier constant rate portion. All values reported were obtained by measuring the slope of the straight line from 0 to 60 minutes.

#### *Cytochrome oxidase*

Cytochrome oxidase was measured following the precautions noted by Stotz (1939). In excess of  $10^{-4}$  mM. per cc. of cytochrome C prepared by the method

of Keilin and Hartree (1937) from beef heart was included with each suspension in measuring cytochrome oxidase activity. As substrates, paraphenylenediamine (ppd) hydroquinone and adrenaline were employed in concentrations of 1/50th molar. In the case of the latter two, a correction for autooxidation was necessary. This was done by including one flask in which all reagents were used as before except that the cell extract was placed in boiling water for 5 minutes. The readings so obtained were subtracted from those of the other flasks. Such corrected readings did not differ from those obtained with paraphenylenediamine which has a negligible rate of autooxidation. Care was taken to keep the tissue content per flask below levels which would yield respiratory rates exceeding 600 cu. mm. per hour when substrate was added.

Within 30 minutes of the addition of substrate from the side arm, a very sharp drop in the rate of oxygen consumption is observed. Five minute interval readings were taken after the introduction of substrate and the calculation of cytochrome oxidase activity is based on the slope of the straight line obtained by plotting the oxygen consumption against time in the 10–20 minute period following the addition of substrate. All the cytochrome oxidase activity measurements on the breis were performed within 60 minutes of their preparation.

#### EXPERIMENTAL RESULTS

Using the methods described, a detailed comparison was made of the respiratory rates of breis and whole eggs during the course of development. The results obtained are summarized by Figure 1.<sup>a</sup> The general exponential nature of the curve describing the variation of respiratory rates of intact embryos during development agrees with those recently published by previous authors (Atlas, 1938; and Moog, 1944). An exception may be noted in the data reported by Barnes (1944) in which a linear rather than exponential rate of rise is observed between stages 12 and 17. Barnes does not discuss this discrepancy between her results and those published earlier by Atlas.

It may be noted in passing that in agreement with Atlas (1938) and Moog (1944) the curve for intact cells in Figure 1 does not rise continuously. A break occurs between stages 12 and 13, a little later in development than the discontinuities observed by the other authors.

The respiration of the brei is high from the very earliest stages of development and remains so well into stage 13 which corresponds to the onset of neurulation. Beyond this stage however it starts to fall, remaining however above the rate of intact eggs in corresponding stages until about stage 17. Beyond stage 18 the respiration rates of the breis are definitely below those of the corresponding embryos. These results confirm the observations of Brachet (1934) who noted that cytolysis of the eggs of *Rana temporaria* during early embryogenesis led to marked increases in the rates of oxygen consumption whereas later in development decreases always followed cytolysis.

It is quite apparent from the respiratory rates of the breis that the eggs contain from the very onset of development sufficient enzymes and oxidizable substrate to support a much higher rate of oxygen uptake than the intact embryos actually do.

<sup>a</sup> The authors are deeply indebted to Mrs. Helen Spiegelman for her able assistance in making the many calculations.

From this result alone it would seem unnecessary to postulate the formation of additional enzyme or substrate to account for the rising respiratory rate during development. The early high rates of the breis can be explained by assuming that the destruction of the cellular structure permits a freer contact of substrate with enzyme than occurs in the normal embryo. The fall in respiration of the brei observed beyond stage 13 can also be explained on the same basis. With the passage of time more and more substrate is consumed resulting in a depression of the brei respiratory rate due to substrate, or possibly carrier, dilution.

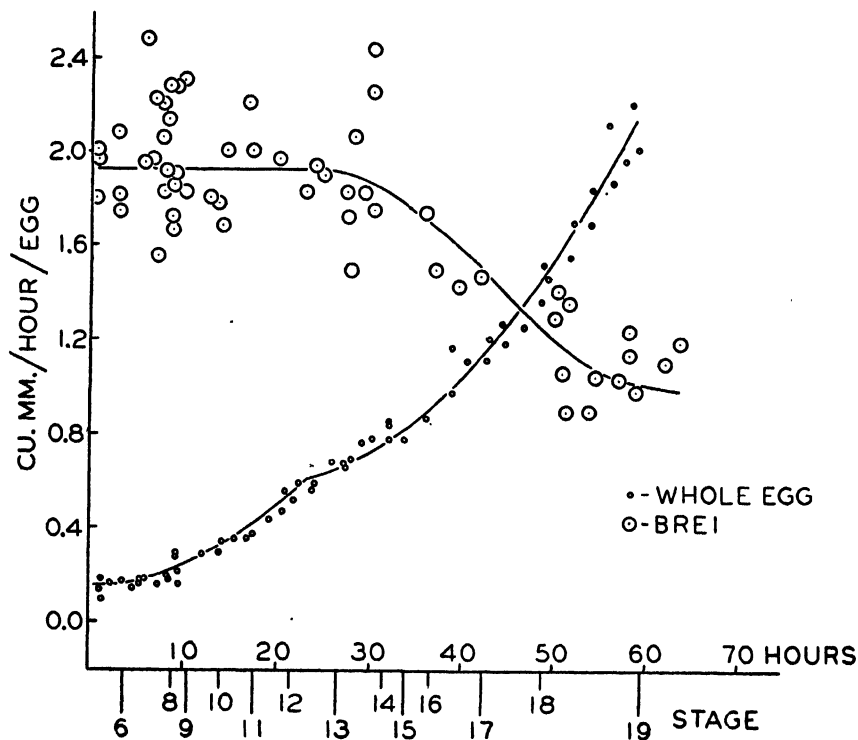


FIGURE 1. Comparison of respiration rates of brei and whole embryo (egg) during the course of development.

### *Response to cytochrome C*

While it was not expected that externally placed carriers could penetrate into the cells, experiments were performed in which very heavy concentrations of cytochrome C, up to  $2 \times 10^{-8}$  mM. per cc., were placed in the external media in which embryos in early stages were suspended. No effect on respiratory rate could be detected.

Since the fall in brei respiration beyond stage 13 might possibly be due to a decrease through loss or inactivation of cytochrome C, this substance was added to breis prepared at various stages of development. Control readings were taken for

approximately  $\frac{1}{2}$  hour before transferring sufficient cytochrome C from the side arms to the main compartment to give a concentration of at least  $10^{-4}$  mM. per cc. This concentration was chosen since it gave maximum rates when substrates of the cytochrome oxidase system were also added. The results are summarized in Table I. It is evident from this table that no significant changes occur on adding cytochrome C. Thus brei respiration is not limited by the content of this factor and the fall in rate beyond stage 13 cannot be ascribed to variations in its concentration.

TABLE I

The effect on respiration rate of adding cytochrome C to brei prepared at different stages of development. All values are based on the cu. mm. of  $O_2$  consumed/egg/hour.

| Stage | Respiration before addition of cyt. C | Respiration after addition of cyt. C | Change |
|-------|---------------------------------------|--------------------------------------|--------|
| 3     | 1.91                                  | 1.90                                 | -0.01  |
| 5     | 2.20                                  | 2.10                                 | -0.10  |
| 6     | 1.85                                  | 1.90                                 | -0.05  |
| 7     | 2.04                                  | 2.04                                 | 0.00   |
| 8     | 2.13                                  | 2.21                                 | +0.08  |
| 9     | 1.92                                  | 1.92                                 | 0.00   |
| 10    | 1.81                                  | 1.87                                 | +0.06  |
| 11    | 2.00                                  | 2.00                                 | 0.00   |
| 12    | 1.92                                  | 1.81                                 | -0.11  |
| 13    | 2.06                                  | 2.00                                 | +0.06  |
| 14    | 1.62                                  | 1.62                                 | 0.00   |
| 15    | 1.72                                  | 1.70                                 | -0.02  |
| 16    | 1.41                                  | 1.30                                 | -0.11  |
| 17    | 1.22                                  | 1.22                                 | 0.00   |
| 18    | 0.93                                  | 1.02                                 | +0.09  |
| 19    | 1.15                                  | 1.20                                 | +0.05  |

#### *Response to addition of cytochrome oxidase substrates*

Paraphenylenediamine or hydroquinone was added in the presence of excess cytochrome C to breis prepared from different stages. Figure 2 summarizes the data obtained. The respiration level following the addition of substrate is, under the conditions of these experiments, proportional to the cytochrome oxidase activity. It is clear from the results that this activity is, as far as can be determined, constant throughout development. Consequently neither the rise in the respiratory rates of the intact embryos nor the fall in the respiratory rates of the breis observed in the later stages can be ascribed to variations in the content of this enzyme. The data obtained with added substrates are consistent with the view that the burst of respiration following cytolysis is due to the breakdown of controls, exerted by geometrical constraint, on existent substrates and enzymes.

It is worthy of note that the addition of the cytochrome oxidase substrates raises the respiration at all stages considerably above that attained by the brei alone. This might indicate that even in the early stages all the available active centers of the enzyme are not completely saturated by endogenous substrate when a brei is prepared.

The results of adding the cytochrome oxidase substrates with or without excess cytochrome C to intact eggs at different developmental stages are summarized in

Table II. For purposes of comparison the same table includes data on the rates of breis following similar additions. For the most part these breis were prepared from the same groups from which individuals were used for the intact embryo experiments.

It is clear that in comparison with the marked increases obtained with the breis, the addition of substrate has relatively little effect on the respiration of intact embryos. No consistent strong increase is obtained at any time from stage 3 up to and including stage 19.

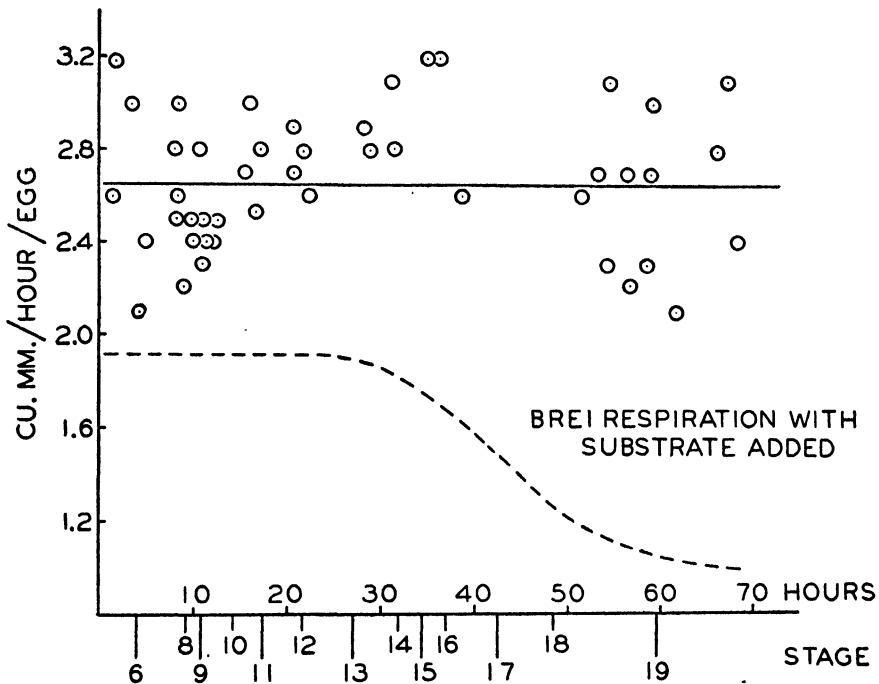


FIGURE 2. The effect on respiration rate of adding substrates of cytochrome oxidase to brei during development. The dotted line represents the curve taken from Figure 1. Single points represent individual experiments.

It is impossible to explain these results on an inability of these substances to penetrate the cells since all of the agents are effective in stopping further development and cause a severe bleaching of the melanin pigment. Both of these facts indicate with some certainty that substrates do penetrate the cells. Thus a simple lack of substrate does not limit the rate of respiration of the intact cells. Carrier immobilization would appear to be the most plausible explanation, although it is conceivable that endogenous substrate somehow forms a protective shell around active centers.

#### EXPERIMENTS WITH INHIBITORS

Assuming that the respiration is largely mediated through the cytochrome oxidase system, it was of some interest to examine the action of inhibitors of cyto-

chrome oxidase. According to Keilin and Hartree (1939) both cyanide and azide are effective against this enzyme.

*Response of brei and intact embryos to NaCN*

In these experiments 0.001 M NaCN was used since it was found by preliminary experiments that higher concentrations did not give significantly greater depressions. The usual precautions suggested by Krebs (1935) were observed and appropriate mixtures of alkali and cyanide were used in the center wells. The results

TABLE II

Respiration (cu. mm./egg/hour) of brei and whole eggs on addition of cytochrome oxidase substrate. Substrate concentration M/50; cytochrome C  $10^{-4}$  mM.

| Stage            | Whole egg | Brei | Whole egg substrate | Brei substrate | Change on adding substrate |      |
|------------------|-----------|------|---------------------|----------------|----------------------------|------|
|                  |           |      |                     |                | Whole egg                  | Brei |
| 3                | 0.21      | 1.6  | 0.20                | 3.2            | -0.01                      | +1.6 |
|                  | —         | 1.7  | —                   | 2.6            | —                          | +0.9 |
|                  | —         | 2.1  | —                   | 3.0            | —                          | +0.9 |
| 4                | 0.21      | —    | 0.20                | —              | -0.01                      | —    |
|                  | 0.20      | —    | 0.20                | —              | 0.00                       | —    |
|                  | 0.25      | —    | 0.21                | —              | -0.04                      | —    |
| 5                | 0.17      | 1.8  | 0.26                | 2.1            | +0.09                      | +0.3 |
|                  | 0.15      | —    | 0.16                | —              | +0.01                      | —    |
|                  | —         | 1.8  | —                   | 2.4            | —                          | +0.4 |
| 7                | —         | 2.2  | —                   | 2.2            | —                          | 0.0  |
|                  | 0.20      | 2.2  | 0.40                | 3.0            | +0.20                      | +0.8 |
|                  | 0.15      | 2.2  | 0.20                | 2.5            | +0.05                      | +0.3 |
|                  | —         | 2.2  | —                   | 2.6            | —                          | +0.4 |
|                  | 0.23      | —    | 0.24                | —              | +0.01                      | —    |
|                  | 0.26      | 1.9  | 0.21                | 3.0            | -0.05                      | +1.1 |
| 8                | —         | 1.7  | —                   | 2.4            | —                          | +0.7 |
|                  | 0.27      | 2.3  | 0.29                | 2.3            | +0.02                      | 0.0  |
|                  | 0.30      | 2.4  | 0.31                | 2.4            | +0.01                      | 0.0  |
| 8 <sup>+</sup>   | 0.18      | 1.7  | 0.26                | 2.8            | +0.08                      | +1.1 |
|                  | 0.20      | 2.1  | 0.22                | 2.4            | +0.02                      | +0.3 |
|                  | 0.20      | —    | 0.23                | —              | +0.03                      | —    |
| 8 <sup>++</sup>  | —         | 1.7  | —                   | 2.5            | —                          | +0.8 |
| 8 <sup>++</sup>  | —         | 2.3  | —                   | 2.5            | —                          | +0.2 |
| 8 <sup>+++</sup> | 0.35      | —    | 0.32                | —              | -0.03                      | —    |
|                  | —         | 1.9  | —                   | 2.5            | —                          | +0.6 |
| 10               | 0.40      | 2.1  | 0.60                | 3.0            | +0.20                      | +0.9 |
|                  | 0.34      | 2.2  | 0.30                | 2.8            | -0.06                      | +0.6 |
|                  | —         | 2.0  | —                   | 2.7            | —                          | +0.7 |

TABLE 11—*Continued*

| Stage             | Whole egg | Brei | Whole egg substrate | Brei substrate | Change on adding substrate |       |
|-------------------|-----------|------|---------------------|----------------|----------------------------|-------|
|                   |           |      |                     |                | Whole egg                  | Brei  |
| 12                | 0.45      | 1.9  | 0.50                | 2.6            | +0.05                      | +0.7  |
|                   | 0.58      | 1.7  | 0.58                | 2.8            | 0.00                       | +1.1  |
|                   | —         | 1.8  | —                   | 2.7            | —                          | +0.9  |
|                   | 0.50      | 2.2  | 0.53                | 2.9            | +0.03                      | +0.7  |
| 12 <sup>+</sup>   | 0.57      | —    | 0.70                | —              | +0.13                      | —     |
|                   | 0.60      | —    | 0.56                | —              | +0.06                      | —     |
| 13                | 0.67      | 2.4  | 0.67                | 3.1            | 0.00                       | +0.7  |
|                   | 0.64      | 2.6  | 0.64                | 2.9            | 0.00                       | +0.3  |
|                   | 0.65      | 2.9  | 0.65                | 2.8            | 0.00                       | -0.1  |
| 13 <sup>+</sup>   | 0.78      | —    | 0.78                | —              | 0.00                       | —     |
|                   | 0.75      | —    | 0.63                | —              | -0.12                      | —     |
| 14                | 0.96      | 1.3  | 0.87                | 2.8            | -0.09                      | +1.5  |
|                   | —         | 1.6  | —                   | 3.1            | —                          | +1.5  |
| 16 <sup>+</sup>   | 1.1       | 1.3  | 0.83                | 3.2            | -0.18                      | +1.9  |
|                   | —         | 1.2  | —                   | 3.2            | —                          | +2.0  |
|                   | 1.2       | —    | 0.94                | —              | -0.26                      | —     |
| 16 <sup>+</sup>   | —         | 1.3  | —                   | 2.5            | —                          | +1.2  |
| 18 <sup>+</sup>   | —         | 0.78 | —                   | 2.2            | —                          | +2.1  |
| 18 <sup>+</sup>   | 1.7       | 1.4  | 1.8                 | 2.6            | +0.1                       | +1.2  |
| 18 <sup>+</sup>   | 2.1       | 1.0  | 2.2                 | 3.1            | +0.1                       | +2.1  |
|                   | 2.1       | 0.90 | 2.6                 | 2.9            | +0.5                       | +2.0  |
| 18 <sup>+</sup>   | 1.6       | 0.98 | 1.2                 | 2.3            | -0.4                       | +1.3  |
|                   | —         | 0.89 | —                   | 2.7            | —                          | +1.8  |
| 18 <sup>+</sup>   | —         | 0.89 | —                   | 2.7            | —                          | +1.4  |
| 18 <sup>12+</sup> | 1.9       | 0.97 | 1.6                 | 2.1            | -0.3                       | +1.1  |
|                   | 2.1       | 0.90 | 1.8                 | 2.7            | -0.3                       | +1.8  |
|                   | —         | 1.00 | —                   | 2.3            | —                          | +1.3  |
| 19                | 2.4       | 1.0  | 2.8                 | 2.4            | +0.4                       | +1.4  |
|                   | 2.6       | 0.97 | 2.0                 | 2.8            | -0.6                       | +1.83 |
|                   | —         | 0.85 | —                   | 3.1            | —                          | +2.25 |

obtained are summarized in Figure 3. The data show that a strong inhibition of whole egg respiration is obtained at all stages. The respiration of the brei, while not diminished to levels quite as low, is also strongly depressed. Figure 4 gives the trend of cyanide sensitivity in terms of per cent inhibition and is based on calculations of average values from Figures 1 and 3. The fall in sensitivity of the brei in

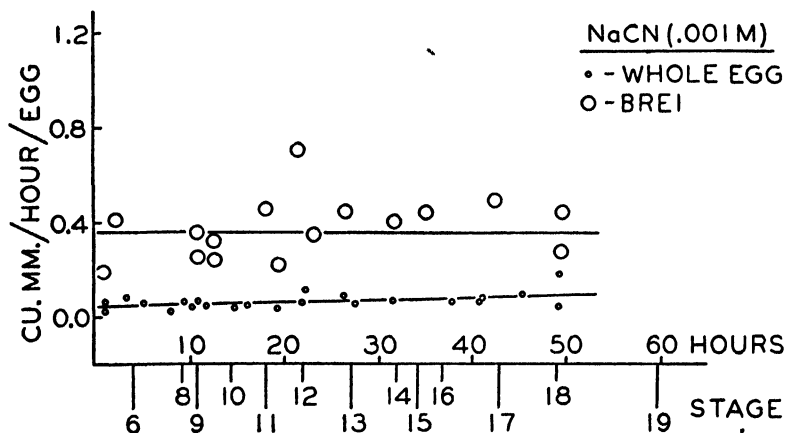


FIGURE 3. Respiratory rate in the presence of cyanide of brei and intact embryos during development.

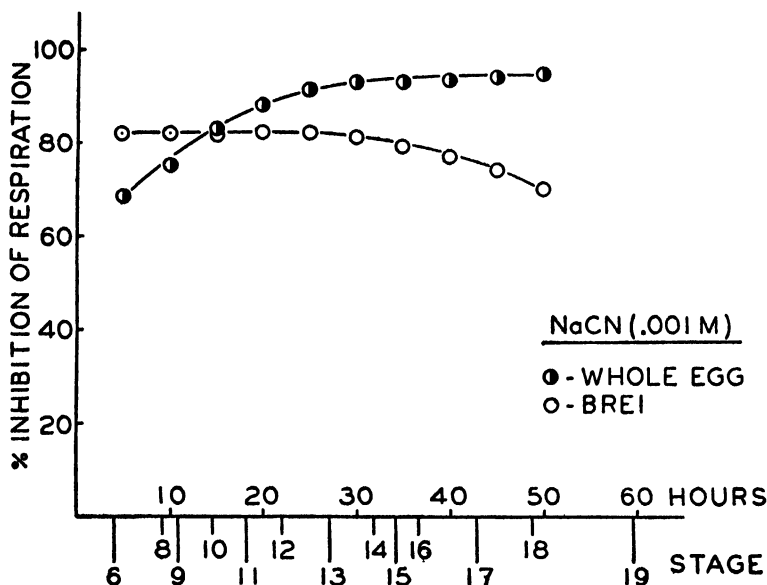


FIGURE 4. Cyanide sensitivity of intact embryo and brei respiration during development.

the later stages is a numerical consequence of the diminishing normal rate during this period which has been attributed to substrate limitations.

These data show that a major portion of the respiration of both brei and intact eggs is mediated via a cyanide sensitive system and that the increments in respiratory rate observed with development are due to the gradual augmentation in the activity of this cyanide sensitive system.

*Response of intact embryos and brei to sodium azide*

The same type of experiments as were carried out with NaCN were also performed with sodium azide at pH 6.7. The concentration selected was 0.005 M since higher concentrations up to and including 0.1 M did not result in more pronounced effect. The respiratory rates of intact embryos and corresponding breis obtained in the presence of azide are given in Figure 5. The percentage inhibitions at the various developmental stages calculated on the basis of averages from Figures 1 and 5 are shown in Figure 6.

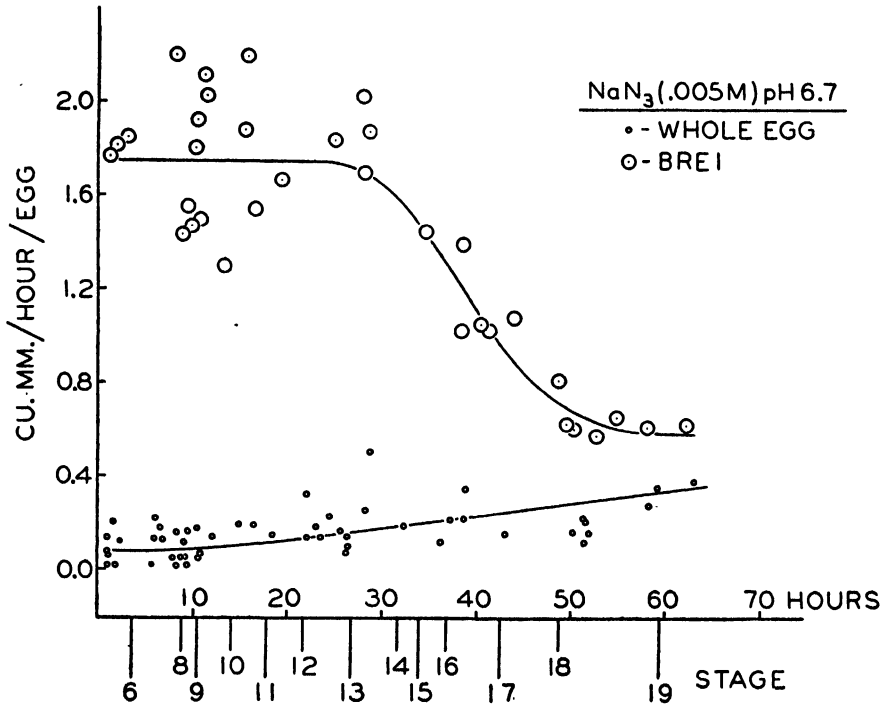


FIGURE 5. Respiratory rates in the presence of azide of breis and intact embryos during development.

In general (Figs. 5 and 6) the behavior of intact embryos towards azide is not very different from that with cyanide. However the residual azide insensitive respiration is consistently greater than its cyanide sensitive counterpart throughout development. Furthermore, unlike the cyanide insensitive respiration which remains constant, the absolute value of the azide insensitive respiration rises during development.

The actions of cyanide and azide on brei are strikingly different. Up until stage 13, 0.005 M azide can decrease the respiration of brei by only 10 per cent. It should be emphasized that this relative ineffectiveness of azide as a respiratory inhibitor against brei is not confined to this concentration only. As may be seen from column 2 of Table III it is true for concentrations as high as 0.1 M.

While Keilin and Hartree (1939) came to the conclusion that azide was virtually equivalent to cyanide in inhibiting the cytochrome oxidase system, recent experiments (Winzler, 1943; Stannard, 1939; Armstrong and Fischer, 1940) show that this conclusion is not universally applicable.

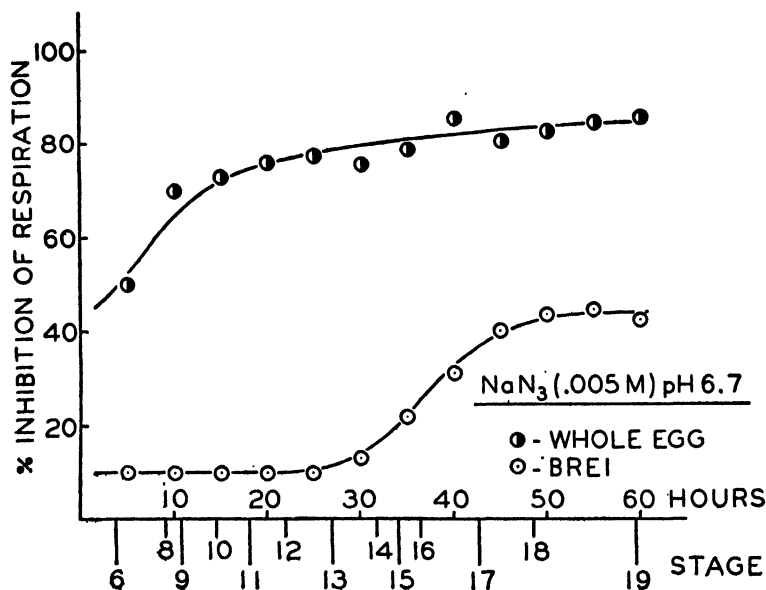


FIGURE 6. Azide sensitivity of intact embryo and brei respiration during development.

To test whether azide attacks the cytochrome oxidase in the breis in the concentrations employed as well as at higher ones, breis were prepared from embryos in stage 9 and placed in the main compartment of Warburg vessels with adequate amounts of cytochrome C and various concentrations of  $\text{NaN}_3$ . Sufficient para-

TABLE III

Effect of various concentrations of  $\text{NaN}_3$  on  $\text{O}_2$  consumption of brei when paraphenylenediamine is added. Rate is cu. mm./egg/hour. All eggs in stage 9.

| M conc. of azide | Rate before substrate addition | Rate after substrate addition | $\Delta$ |
|------------------|--------------------------------|-------------------------------|----------|
| 0.000            | 1.91                           | 2.51                          | +0.60    |
| 0.005            | 1.70                           | 2.29                          | +0.59    |
| 0.01             | 1.80                           | 2.50                          | +0.70    |
| 0.05             | 1.63                           | 1.75                          | +0.13    |
| 0.10             | 1.65                           | 1.67                          | +0.02    |

phenylenediamine was placed in the side arms to yield a final concentration of 1/50th molar. After an adequate number of readings were taken to establish the rate without substrate, the substrate was introduced and the resulting respiration followed.

The results are given in Table III. Concentrations up to and including 0.01 M are unable to prevent the rise which occurs on adding substrate. The two higher concentrations tested, 0.05 and 0.1 molar, although ineffective against the respiration of the endogenous substrate, were capable of preventing the burst which normally follows the addition of paraphenylenediamine.

Similar experiments were carried out with cyanide (Table IV). Cyanide not only depresses the brei respiration to levels previously noted but in addition prevents any rise when the paraphenylenediamine is added.

TABLE IV

Effect of various concentrations of NaCN on  $O_2$  consumption of brei when paraphenylenediamine is added. Rate is cu./mm./egg/hour. All eggs stage 9.

| M conc. of NaCN | Substrate rate before addition | Rate after substrate addition | $\Delta$ |
|-----------------|--------------------------------|-------------------------------|----------|
| 0.000           | 2.14                           | 2.86                          | +0.72    |
| 0.001           | 0.51                           | 0.51                          | 0.00     |
| 0.002           | 0.32                           | 0.38                          | +0.06    |
| 0.003           | 0.24                           | 0.24                          | 0.00     |
| 0.005           | 0.44                           | 0.54                          | +0.10    |

These experiments with cyanide establish with some degree of certainty that the normal brei respiration is mediated by the Warburg-Keilin system. At least three important criteria for this assertion are satisfied: (1) The system exists in the brei. (2) The response to cytochrome oxidase substrate is cyanide sensitive. (3) The respiration of endogenous substrate is also cyanide sensitive. It is therefore necessary to assume that insensitivity of the brei respiration to azide is due to the latter's inability to combine with the cytochrome oxidase in the brei. It is conceivable that azide cannot combine readily with the cytochrome oxidase when the other enzymatic components and substrates of the cell are present in the extract.

It is certainly reasonable to expect that the sensitivity of a given enzyme system to a particular inhibitor will depend on the kind and amount of the substrate with which it is competing for active centers. It is therefore not surprising that cytochrome oxidase should be more sensitive to azide when in comparative isolation. That some such competitive mechanism is working may be seen from an analysis of the data in Table III and in particular from the fact that the two highest concentrations employed, while leaving the respiration of endogenous substrate relatively untouched, effectively suppress the increased oxidation when paraphenylenediamine is added. These findings would be expected if at the higher concentrations of azide the inhibitor could compete successfully for an active site on the enzyme surface with a molecule of paraphenylenediamine but not with a molecule of endogenous substrate. Further, it will be recalled (see Fig. 6) that azide sensitivity of the brei respiration starts to rise beyond stage 13 when the normal rate starts to fall due to substrate limitations. This finding would also be expected, since with substrate concentration falling the ratio of azide to endogenous substrate rises and the azide can then start to compete successfully for active centers.

While the above interpretation fits all the data obtained on the relative azide insensitivity of brei it leaves unexplained the effective inhibitory action on intact embryos shown in Figure 5. If we interpret this inhibition as a reaction between the Warburg-Keilin system and azide we would necessarily conclude that this agent can combine with cytochrome oxidase in intact eggs but not in cell extracts. A plausible reason for this difference may be seen by focussing attention on substrate availability in the two situations. Here again we probably have a situation analogous to the increasing azide sensitivity of brei when the rate is falling i.e., less severe competition by endogenous substrate molecules for active centers as their concentration decreases. It is undoubtedly true that in the intact eggs the number of substrate molecules getting to the enzyme surface is severely regulated, permitting the azide molecules to compete successfully.

It is possible to explain the different responses of brei and whole egg to azide by assuming that in neither case can the azide combine with cytochrome oxidase in the concentrations employed. The capacity of azide to inhibit synthetic processes has been demonstrated for certain systems (Winzler et al., 1944; Moog and Spiegelman, 1942). Most important for the present discussion is the finding by Spiegelman and Moog (1945) that azide completely inhibits amphibian development at all stages including those between fertilization and gastrulation. From this point of view then, azide may depress the respiratory rates of intact eggs indirectly because it inhibits synthetic activities leading to substrate availability for enzyme action.

#### DISCUSSION

The data presented find their most plausible interpretations in terms of structure orientation and the consequence of its destruction. The comparison of brei and intact embryos in the course of development as well as the determination of cytochrome oxidase content ruled heavily against enzyme or substrate synthesis as explanations for the rising respiratory rate during development. This emphasis on the internal geometry of enzyme and substrate and its variation as determining factors is a point of view that is becoming increasingly popular. Recent reviews by Korr (1939) and Commoner (1942) have stressed it as a criterion for evaluating data obtained from *in vitro* experiments. Both Runnstrom (1930) and Korr (1937) have discussed the sudden activation of the Warburg-Keilin system on fertilization of Arbacia eggs in terms of the relative positions of enzyme and substrate. Ballentine (1940a, b) who studied the dehydrogenase activity in the same material suggested similar considerations for these enzyme systems. It seems inescapable that more refined interpretations of physiological processes must consider not only what enzymes and substrates exist in the cell, but also where they exist. The present investigation which explains rising respiratory rate in terms of reorientation of existent components indicates that this is equally true for embryonic processes.

#### SUMMARY

The respiratory rates of whole embryos and cell-free breis were determined at various stages of development. During early stages, when the respiration of whole

embryos is low, brei respiratory rate is high, the situation being reversed beyond about stage 17.

Cytochrome oxidase remains uniformly high throughout development as shown by the effects of adding substrate to breis. Externally applied cytochrome oxidase substrates have little effect on intact embryos although the agents are shown to penetrate. Cytochrome C is shown not to be a limiting factor in brei respiration.

Cyanide depresses the respiration of both breis and embryos. Azide has little effect on respiration of breis but is very effective in depressing oxygen consumption and development of intact embryos.

The results are discussed in terms of spatial orientation of enzymes and substrates.

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# PINNOTHERES OSTREUM, PARASITIC ON THE AMERICAN OYSTER, OSTREA (GRYPHAEA) VIRGINICA<sup>1</sup>

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## INTRODUCTION

The oyster crab, *Pinnotheres ostreum*, was first described by Say in 1817. It is an organism known for many years to oystermen and biologists alike although its habits and life history are but imperfectly known. In her monograph on the grapsoid crabs Rathbun in 1917 defined the adult characters of the genus and compared the numerous species which had been collected from many parts of the world in association with various mollusca, tunicates, annelids and sea urchins. This association has regularly been considered commensalism by most investigators. In the case of *P. ostreum*, however, the relationship is not clear for in 1892, Bashford Dean wrote that the crab was evidently annoying to the oyster for the palps sometimes show thickened out-growths or are malformed and stunted in size. Orton, in 1921, presented clear evidence in the case of the pea crab, *P. pisum*, from the mantle cavity of the mussel, *Mytilus edulis*, that the activity of the crab was definitely parasitic in nature. Ryder, on the other hand, supposed that the crab was of value to the oyster since the latter was said to feed on the clusters of bell-animalcules (*Zoothamnium* sp.) which are attached to the crab's shell. A consideration of the crab's stomach contents, however, showed that its food consists in great part of the minute organisms sought by the host in addition to small crustaceans not normally the oyster's prey.

A sudden increase in the numbers of *P. ostreum* in Delaware Bay in 1941 associated with definite erosions of the gills of the oyster and producing, or at least contributing to, the death of many oysters stimulated a re-examination of the crab. The data obtained (abstracted recently, Stauber, 1942) are the subject of this paper.

## LIFE HISTORY

Atkins (1926) has described the post-planktonic stages in the developmental cycle of *P. pisum* from *Mytilus edulis* in British waters. The situation with *P. ostreum* is so similar that, except for morphological details, it seems to be the same. The invasive stages of the oyster crab are small, hard, flat crabs with a carapace 1.4–3.4 mm. wide (Figs. 1, 3, and 23). The sexes are separate and indistinguishable except for the number of pleopods and the genital apertures. Apparently copulation takes place after invasion of the oyster and perhaps is necessary, as Atkins suggests, in order for further development of the female crab to take place. In *P. pisum* she

<sup>1</sup> The author wishes to express his deep appreciation for the many ways in which Dr. Thurlow C. Nelson aided and inspired this work.

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found that in all other stages of the female crabs the spermathecae contained some spermatozoa. Occasionally only one spermatheca was full but more often both were distended with sperms. Except for a few males similar in external appearance to the second stage females no other stage of the male *P. ostreum* is known and the male is believed to be free-swimming like the invasive first stage female.

The second stage female differs markedly from the first stage being rounder in form, grey in color with prominent chromatophores, with a softer carapace and slenderer legs. In size, however, it measures only slightly larger (0.9–3.1 mm.).

The third stage female is still larger (2.6–4.4 mm. wide) but aside from this is quite like the second stage in color, shape and appearance. Higher development of the pleopods differentiates it from the latter.

The fourth stage female is pale yellow in color and is again larger (3.6–8.9 mm. wide).

The adult female, or fifth stage crab, ranges in size from 6–14.9 mm. and is the form of *P. ostreum* commonly known. Indeed, except for this stage, only a few males and none of the other female stages have ever been recorded. Several moults of the fifth stage female crabs are possible and there is some overlapping of sizes of the crabs in various stages due to time of invasion, food supply, size of the oyster invaded, etc. No data are available on the rate of growth or time lapse involved between the various female stages. Only moults of fifth stage females and one moult of a third stage female into the fourth stage have been obtained in the laboratory. In one dredged oyster a fifth stage female and a moulted shell were obtained within the same bivalve.

Ovigerous females are seen in the summer and autumn of the year and Atkins believes that *P. pisum* may become adult in a single year. There is reason to believe that such is the case with *P. ostreum* also. Judging by the size of the mature female crab, with reference to the male copulatory pleopods, it seems unlikely that a second copulation takes place. Therefore, if the large crabs live more than one year, which is quite probable, they must have received sufficient sperm in the one copulation to last for several batches of eggs or become capable of producing only infertile eggs. Orton and also Atkins found the majority of large females of *P. pisum* to have full spermathecae but an occasional large female with empty spermathecae led the latter to postulate that copulation may occur more than once.

After a time, the length of which is still unknown, the developing embryos hatch from the eggs attached to the pleopods of the mother producing a zoea which has been described for *P. ostreum* by Birge (1882) and again by Hyman (1924). In our own experience an ovigerous female was obtained from an oyster dredged as late as October 19, 1942. The embryos then were almost ready to hatch and showed a pulsating heart, a fully formed abdomen, eyes, a digestive gland, and appendages. Hatching began four days later and large numbers of the first zoea were available for study.

Subsequent planktonic stages of this crab are still unknown but judging by Lebour's (1928) criteria (the relatively primitive condition of the first zoea) three or more zoeal stages are probably present and possibly also a megalopa. Eventually, however, the free-swimming invasive male and female crabs are formed completing the cycle.

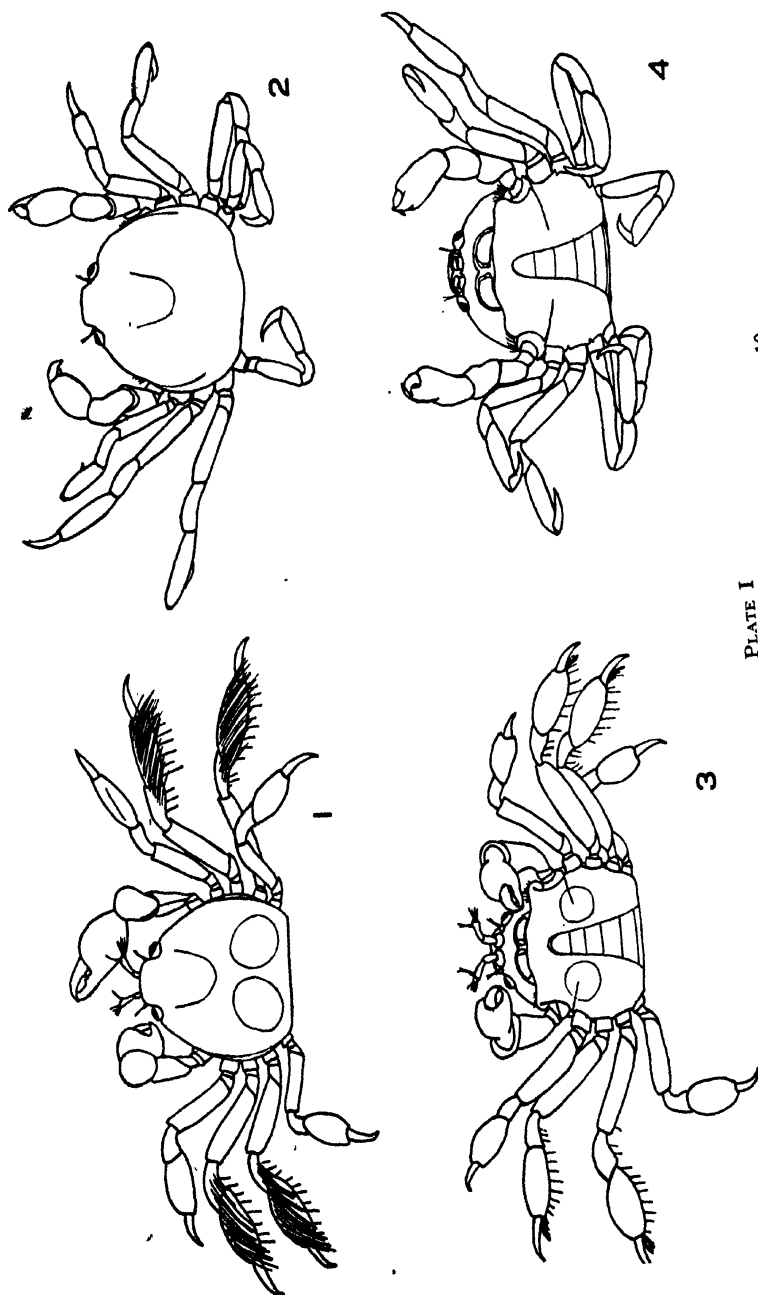


PLATE I  
First and second stage female *Pinnotheres ostreum*.  $\times 10$

FIGURE 1. Dorsal view of Stage I female.  
FIGURE 2. Dorsal view of Stage II female.  
FIGURE 3. Ventral view of Stage I female.  
FIGURE 4. Ventral view of Stage II female.

## MORPHOLOGY OF THE VARIOUS STAGES OF THE CRABS

*The male*

In contrast to *P. pisum*, the male *P. ostreum* has been considered an unusual find, Rathbun (1917) reporting only two specimens in the National Museum collection. In size it is much more restricted than the male *P. pisum*, the carapace usually being from 1.5–3.4 mm. wide with sizes below 1.6 and above 2.5 mm. being rarely seen. The mean carapace width of 144 specimens was  $2.13 \pm 0.19$  mm. Furthermore, unlike all but the first stage female from which it is almost undistinguishable, its shell is well fortified with calcium salts probably making it as hard for its size as species of free-living crabs. In color it is dark brown with two large distinct almost circular pale white spots, visible both on carapace and on the sternum. On the posterior half of the dorsal side they are situated in the branchial regions. On the ventral side they flank the groove which contains the abdomen and are situated at the level of and medial to the first pair of pereopods or walking legs. In a male, whose carapace was 2.0 mm. wide the dorsal spots were roughly 0.6 by 0.5 mm. On the ventral side of the same crab the pale areas were only 0.45 mm. in diameter. The carapace is flat dorsally, sub-circular in shape with an advanced, more truncate front than is present in the later females. The posterior margin is straight and the widest part of the carapace about  $\frac{4}{7}$  the distance from the anterior end. The eyes are well developed. The front is about  $\frac{3}{4}$  as wide as the carapace. The lateral margin of the carapace is thin and rather sharply bent from the dorsal side. Antennules are large and antennae small. The epistome is well defined. The buccal cavity is crescentic, arched and very broad from side to side but very short fore and aft. The external maxillipeds completely close the cavity and consist chiefly of the merus fused with the ischium and with the flagellum attached to the inner end. Carpus of the first segment of the palp or outer maxilliped, short, oblong; propodus more elongate, rounded; dactylus inserted behind middle of the propodus, minute and slender. Chelipeds (Fig. 5)\* are stout, merus and carpus not slender as in the later females. Propodus slightly flattened inside, swollen outside and strongly widened from proximal toward distal end, narrowing again so that width at base of dactyli is considerably less than the greatest width of propodus. Both margins of propodus are convex. Fingers are stout, especially the immovable one with the tips hooked past each other when closed. The movable finger has a small tooth which fits between two protuberances on the immovable finger. Stiff hairs project from the gripping surfaces of both fingers.

The pereopods are markedly flattened with the propodi almost spatulate, the third leg being slightly longer and stouter (Figs. 6–9). The propodi are widest about centrally, being half as wide as long and over twice as wide as thick ( $0.42 \times 0.18$  mm. in one case). The posterior border of the propodi of the walking legs is

\* Although Figures 5–9, inclusive, were drawn specifically from a first stage female crab, the respective appendages of the same stage male are indistinguishable.

## PLATE II

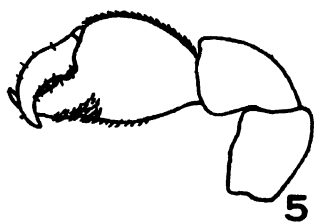
Ventral view of chelipeds and pereopods of Stage I and II female *Pinnotheres ostreum*.  $\times 54$

FIGURE 5. Cheliped of Stage I female.

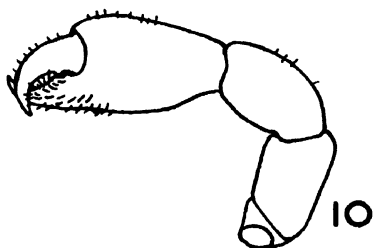
FIGURES 6–9. Pereopods of Stage I female.

FIGURE 10. Cheliped of Stage II female.

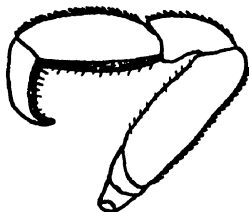
FIGURES 11–14. Pereopods of Stage II female.



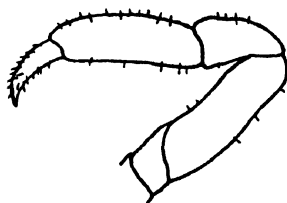
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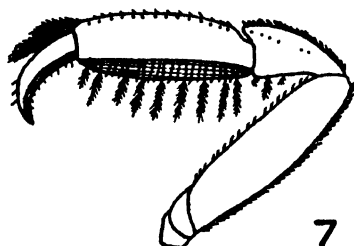
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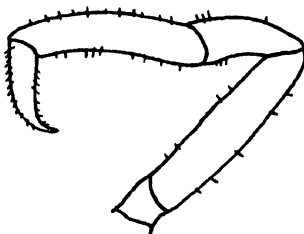
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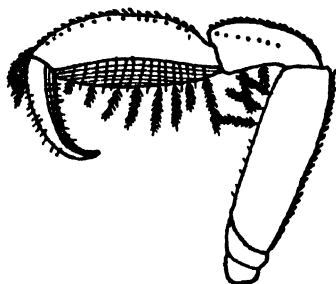
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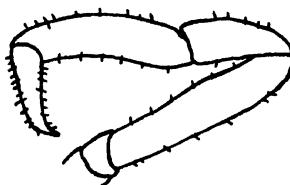
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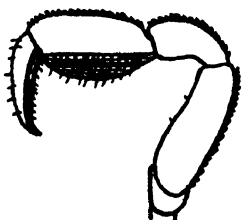
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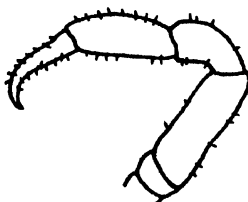
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13



9



14

thickened. The amount of thickening is progressively greater from first to fourth walking leg, being not quite half the total width of the propodus of the fourth leg. The thickened portion seems to be constituted of the same material as the rest of the exoskeleton. Not only does it show longitudinal striations as though produced by successive depositions of chitin but the whole thickness is pierced by the hairs which protrude from the posterior border of each of the propodi. The dactyli of the same legs also show a similar thickening of the posterior which is likewise pierced by the protruding hairs. Unlike the female, the dactylus of the third leg of the male is longest but it is not markedly longer than that of the second which is almost as long and a trifle more curved at the tip. Also unlike the adult female, the dactyli of the second and third legs are only slightly more or less than half the length of the propodus of the same leg. There are two rows of long plumose hairs on the second and third legs. One row extends postero-dorsally from the anterior edge of the dorsal surface in a line running from midway on the carpus to the distal end of the propodus. Some of the hairs are over 0.6–0.9 mm. long. The other row extends backward from the posterior border of the flattened leg also extending from middle of the carpus to distal end of the propodus. These hairs are only about half as long as those on the anterior edge. The other joints of these legs and the other legs only possess short hairs or setae which are stout and plumose and are especially abundant on the anterior edges of the propodus, carpus and merus. We consider that these flattened, hairy legs are adaptations for free-living existence. Indeed, the first stage crabs are fair swimmers and reach the oysters by this means. The sides of the abdomen are almost straight and become narrower from the third to the seventh segment (Fig. 16). The terminal segment is arcuate. The abdomen fits neatly into the grooved sternum. A locking apparatus, somewhat like that described by Atkins for *P. pisum*, is present in the male *P. ostreum* (Figs. 15 and 16). It consists of a pair of almost conical chitinous knobs (on the fifth thoracic segment) along the side of the grooved area where the abdomen fits and a pair of larger blade-like protuberances on the sixth segment. Both sets of knobs project slightly anteriorly, ventrally, and medially.

Instead of fitting into grooves on the abdomen there are shelf-like projections on the fifth and sixth abdominal segments. The shelf on the fifth segment is much shallower and smaller than that on the sixth. The knobs on the thorax are hooked under these shelves making a close-fitting efficient locking mechanism that can easily be undone by the crab through extension of the abdomen and lifting but which is

### PLATE III

#### Some details of structure of *Pinnotheres ostreum*

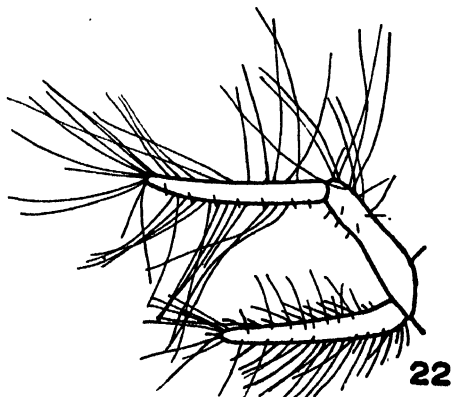
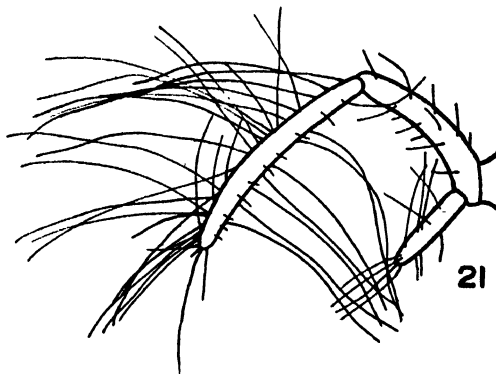
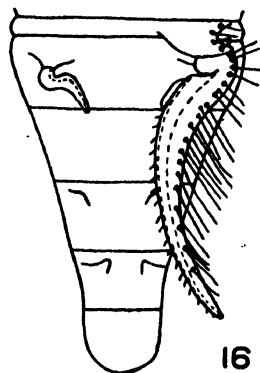
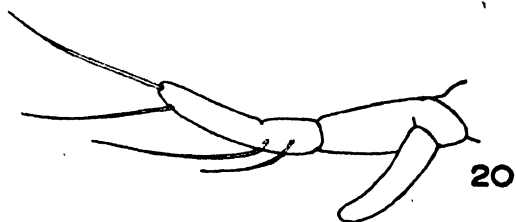
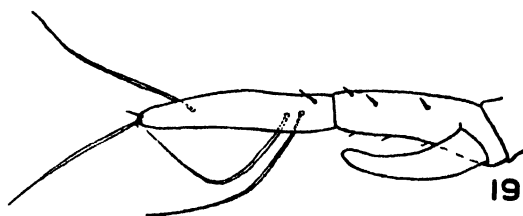
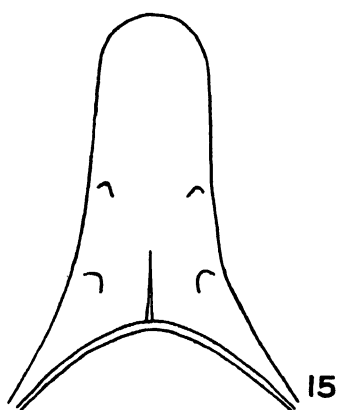
FIGURE 15. Ventral view of the sternum of a male crab (Stage I) showing the chitinous knobs of the locking mechanism.  $\times 20$ .

FIGURE 16. The abdomen of a Stage I male crab showing the copulatory pleopods. The first appendage on the right side was removed to better expose the smaller second appendage. Figure also shows the shelves of the locking mechanism.  $\times 20$ .

FIGURES 17 AND 18. First and second pleopods, respectively, from the left side of a Stage II female crab.  $\times 166$ .

FIGURES 19 AND 20. First and second pleopods, respectively, from the left side of a Stage III female crab.  $\times 43$ .

FIGURES 21 AND 22. First and second pleopods, respectively, from the left side of a Stage IV female crab.  $\times 22$ .



difficult to dislodge in the living animal where the stronger the flexing strain the more tightly the knobs lock.

The only thoracic somite dividing suture on the sternum is seen extending mediad from behind the coxa of the cheliped to a point a little more than midway to the groove where the abdomen lies. This is between the fourth and fifth thoracic segments. The copulatory organs of the male are large, like those in *P. pisum*, the first is blade-like and hairy and the second is rod-like (Fig. 16) and almost hairless. The tube runs along the medial side of the first appendage and the second appendage fits into a groove running across the under surface of the first appendage near the base of the terminal segment. The first appendage is more slender and scimitar-like than the same appendage on *P. pisum*.

### *First stage female*

This is the female stage which invades the oyster (Figs. 1 and 3). It is superficially indistinguishable from the male although it is usually slightly smaller on the average (1.4–2.4 mm.). The mean of 187 measurements of width was  $1.83 \pm 0.14$  mm. Not until careful examination is made of the abdomen and the sternal groove into which it fits can differentiation be made.

For example, there is only one pair of chitinous knobs (corresponding to the anterior pair of the male) and consequently only the terminal or corresponding pair of shelves on the abdomen (on the sixth segment). The genital aperture is small and located just posterior and medial to the base of the chitinous knob on each side. The relationship is thus almost precisely like that in *P. pisum*. The abdomen in the female has slightly straighter sides with the terminal segment having a lower arch. It lacks one pair of locking shelves and the segmentation between 3 and 4 and between 4 and 5 is much less distinct than in the male. Furthermore, the first segment is widest unlike the male where the third segment is widest. Such differences are very minor and usually cannot be determined *in situ* but only by removing the abdomen for comparison. Then, however, these differences are quite constant. Finally, the abdomen of the female bears four pairs of minute appendages, the first one of which is biramous, the second has a bilobed tip, presaging its eventual biramous state, and the other two are simple knobs. The walking legs are like those of the male.

### *Second stage female*

This stage hardly exceeds in size the range of the first stage female. The carapace width of 67 specimens ranged from 0.9 to 3.1 mm. (mean  $2.1 \pm 0.23$  mm.). The moult which occurs involves more differentiation than growth. The carapace is now smooth, shining, thin and membranaceous and, unlike the previous stage, yielding to the touch. It is convex from before backward and the lateral margins are thick and bluntly rounded. The front is still about  $\frac{2}{3}$  the width of the carapace but now it is less truncate and does not project quite so far forward (Figs. 2 and 4).

The chelae are slightly more slender with the propodus widest more distally, nearly at the base of the fingers (Fig. 10). The pereopods (Figs. 11–14) are slender and subcylindrical with delicate hairs on the last three segments. No such plumose hairs are present as described on the Stage I crabs. Propodus is not flattened on pereopods as in Stage I nor does it broaden out very much, being only

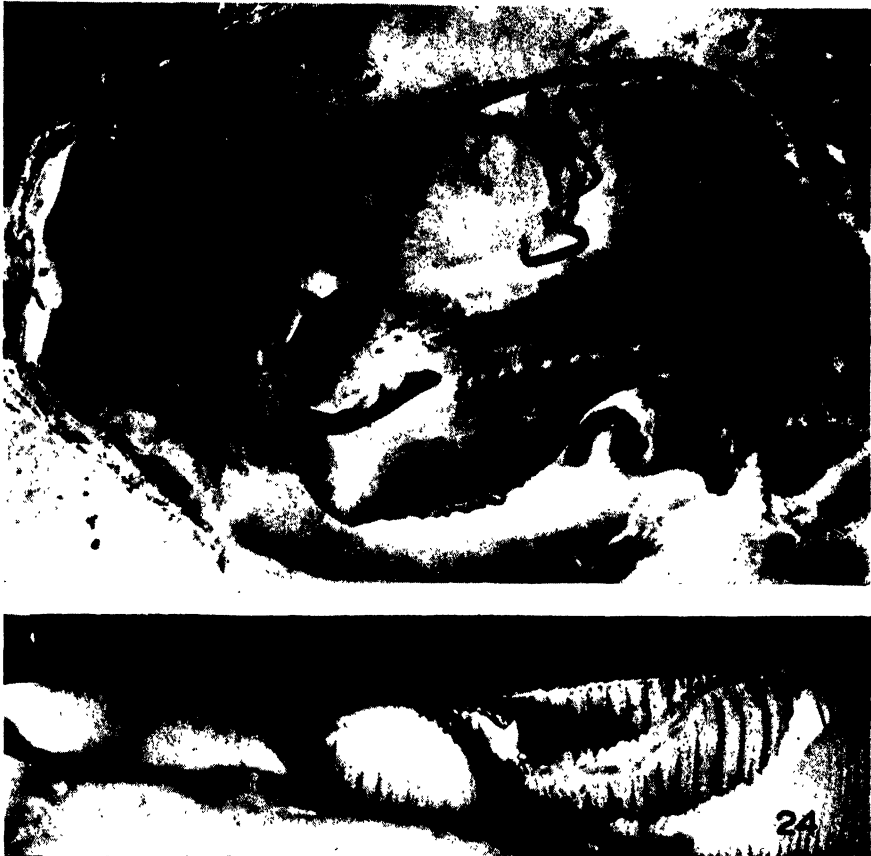


PLATE IV

Photomicrographs of *Pinnotheres ostreum* and gill-eroded oysters. I wish to express my appreciation to Dr. T. C. Nelson for these. He not only took the photographs but found the particular specimens displayed. All  $\times 3$ .

FIGURE 23. View of large-crab type gill erosions in an oyster. Note the presence of both a large Stage V female crab and a small Stage I male crab. The demibranchs of the oyster are shorter than normal and the ventral mantle cavity much enlarged.

FIGURE 24. Small-crab type erosions of gills. Note the sharply-delimited, punched-out area involving three demibranchs.

slightly wider than the merus of the same leg. The third leg again is largest and the dactyli of second and third legs nearly equal in length.

The abdomen is approximately the same shape as in the first stage. The ratio of carapace width to abdomen width is 2.46 and of abdomen length to abdomen width is 1.36. The first is still the widest segment. The terminal segment is only slightly wider ( $1\frac{1}{2}$  times) than long in this stage. The sternum is still deeply grooved to hold the abdomen flush. Chitinous knobs are still to be seen on the thorax but the mechanism is now weakened by the delicate nature of exoskeleton.

This is obvious the first time one attempts to lift the abdomen. In Stage I males and females this is accomplished with difficulty and often the abdomen is injured in the process. In this and subsequent stages lifting of the abdomen is easy. Here is an adaptation to the sheltered life within the mantle cavity of the oyster which free-living first stages do not show. The legs also, no longer needed for swimming, have become slender and relatively smaller with each stage of development.

The abdominal appendages are but little advanced over those seen in the first stage female though in some instances they seem farther along than others. This and subsequent stages, while rather clearly defined, do vary quite broadly within the stage. In some there seems to be little change over the appendages of the first stage female, in others the biramous character of the first two pairs is more clearly marked. The appendages are still hairless, small and relatively undifferentiated (Figs. 17 and 18). There are many chromatophores scattered over carapace, sternum and abdomen. The legs have few of these black chromatophores although the chelae are well supplied. Consequently, the crab in this stage, against a light background, appears mottled grey with translucent appendages. Against a dark background, the appendages show up better and appear opaque. The later stages, especially the third stage female, are quite similar in color but cream-colored specimens are seen in increasing abundance. This is due chiefly to the decrease in numbers of these chromatophores and dispersion of pigment within them, and an increasing opacity of the organs within the crab. The adult female is always cream-colored except for the reddish outline of the mature gonad, the anterior parts of which are seen through the carapace from above and the posterior parts through the abdomen from below.

#### *Third stage female*

The chief differences are those of size, shape, and relative size of abdomen and character of the abdominal appendages. The width is from 2.6–4.4 mm. (mean of 31 specimens was  $3.17 \pm 0.40$  mm.). It was found less frequently than first or second stage crabs in 1941 but has been relatively more abundant in 1942. The second and third walking legs are nearly equal in length with the dactyli also almost the same length. The average ratio of carapace width to abdomen width in eleven specimens was 2.0 and that of abdomen length to abdomen width was approximately 1.1. Thus the width of the abdomen has relatively increased slightly over both the length of the abdomen and the width of the carapace. In most of the specimens the third or fourth segment was widest. The terminal segment of the abdomen now has a flatter arch, usually being over twice as wide as long. The abdominal appendages, especially the first two pairs, are clearly segmented and possess a few distinctly plumose hairs (Figs. 19 and 20). The edges of the abdomen now extend beyond the broadened and shallower depression in the sternum which in earlier stages wholly contained the abdomen. There are hairs on the lateral edge of the abdomen in this stage. These hairs are more abundant on the more proximal segments and are quite variable in number. They are stout, plumose and rarely exceed 0.15 mm. in length.

#### *Fourth stage female*

Specimens on hand range in size from 3.6–8.9 mm. The abdomen is much broader now and just reaches the coxa of the legs in the majority of specimens.

It extends forward almost to the posterior edge of the mouth. The sternal depression is much broader in this stage though the abdomen overlaps it considerably. The third or fourth abdominal segment is widest and the abdomen wider than long even when pressed out flat. The carapace-abdomen width ratio has also further decreased and is now nearly equal to 1.0. The terminal abdominal segment has broadened to such an extent that it is now three to five times as wide as it is long. The abdominal legs are almost fully developed and well supplied with hairs (Figs. 21 and 22). The hairs along the lateral edge of the abdomen are more numerous and large and are present on all segments as a uniform fringe in the fourth stage crab.

The orbit has hardly increased in size and the diameter of the eye is practically the same. Thus the relative prominence of the eyes has decreased markedly. The carapace is now much more convex so that the front appears to be ventral in position. The abdomen is very convex externally and cannot be flattened without tearing the exoskeleton. Ventrally the hollowed abdomen is suited for carrying the masses of ova.

#### *Fifth stage female*

This is the stage commonly seen. The prominence of the reddish gonads identifies it in most cases though some fifth stage females are found with immature gonads. A few apparently fourth stage crabs show mature gonads, however. The features which differentiate the fourth and fifth stage females are rather slight. The abdomen is now wider than the carapace, extending to the basis of the walking legs in many cases. The terminal segment is more than five times as wide as long. The abdomen projects forward to a point anterior to the mouth which it covers except when feeding. For this act it rolls inward the terminal segments exposing the mouth parts as described for *P. pisum* by Orton. The size ranges from 6.0–14.9 mm. (34 specimens measured). It is possible that adult size is determined in part, as Atkins found for *P. pisum*, by the size of the host invaded. Even here, however, the range is great. Thus, a fifth stage crab 9.0 mm. wide was found in an oyster only 29 mm. long. Its abdomen was 12 mm. wide and extended beyond the coxopodites. No studies relating host size with crab size have been made but such a factor would account partly for the overlapping sizes of the various stages of the female crab. Another factor would be subsequent moulting after the fifth stage is reached. This is known to happen and several fifth stage females have moulted in the laboratory. Since only one moult of an earlier stage has been obtained which resulted in the production of a fourth stage female from a third stage crab, it is not known whether all earlier moults involve metamorphosis or whether growth moults can occur between stages. Biological variability and amount of available food are probably additional factors in size variations.

The walking legs of the adult female are different in their proportions from the earlier stages for the second leg is distinctly longer than the others and the dactylus of the second leg is more than two-thirds as long as the propodus. In our specimens, however, it does not seem to be regularly seven-eighths as long as Rathbun described it.

Two ovigerous females are present in our collection, the first measures 14.0 mm. across the carapace but the other only 8.9 mm. This is a further indication of the variability existing in the stages of the crab.

*Second stage male*

The presence of a number of specimens similar in external appearance to second or third stage females (body shape, exoskeleton yielding to the touch, slender, translucent legs, and grey or cream in color) has been noted. Thirteen measured specimens averaged 3.1 mm., ranging from 2.0-4.8 mm. Shape of abdomen and presence of typical male pleopods clearly identify these crabs as males. The pliable abdomen, however, only has one pair of the shelves of the locking mechanism though the sternum still shows both pairs of chitinous knobs. Like the females (except those of Stage I) the exoskeleton is delicate in these soft-bodied males and the abdomen can be easily lifted from the sternum even in living crabs. Since these crabs have been found in appreciable numbers it is quite possible that they may serve as partners in copulation with larger females of later stages though no evidence is available for or against this possibility. The size range would indicate that at least one, and probably two, moults can occur beyond the typical hard-shelled first stage males. It is also possible that these atypical males are the result of some sort of parasitism as Mercier and Poisson (1929) have reported for *P. pisum*. They found that males parasitized by the entoniscid, *Pinnotherion vermiforme*, resembled immature females in increased size and decreased firmness of the carapace and in the diminished number of hairs and greater slenderness of the legs. Parasitized female *P. pisum* were not perceptibly modified in these characteristics.

## PRESENCE OF PINNOTHERES IN OYSTERS

All the stages of the crab described in the preceding pages were found in oysters from Delaware Bay in both 1941 and 1942. Although multiple infestations occurred involving various combinations of the crab stages, no case has been found containing more than a single specimen of either the third, fourth, or fifth stage crabs. First stage males and females were in great abundance in 1941 but were much less frequently seen in 1942 either per oyster opened or proportionate to the other crab stages. This is indicated in Table I where the same grounds of oysters were sampled in the two years. The only differences were that the oysters in each case were a year older in 1942 and that a twenty-five to thirty-three per cent mortality had occurred on each ground during the intervening winter.

TABLE I

*Distribution of numbers and stages of P. ostreum in oysters from two grounds sampled in consecutive years*

| Ground | Year | Mean no. of crabs per oyster* | Max. no. of crabs per oyster | Oysters with crabs | Stage I crabs | Stage V crabs |
|--------|------|-------------------------------|------------------------------|--------------------|---------------|---------------|
| A      | 1941 | 6.0                           | 18                           | 80%                | 91.4%         | 1.2%          |
|        | 1942 | 1.1                           | 3                            | 73%                | 41.7%         | 33.3%         |
| B      | 1941 | 4.2                           | 19                           | 80%                | 92.0%         | 1.6%          |
|        | 1942 | 1.1                           | 5                            | 55%                | 50.0%         | 30.0%         |

\* Based on total number of oysters in the sample.

We offer the data in this table also as additional evidence in favor of Atkins' belief (although she was working with *P. pisum*) that growth to maturity takes place within a year's time. The marked increase in both absolute and relative numbers of Stage V crabs in relation to a marked decrease in total crab population is more than suggestive.

The greatest number of crabs in a single live oyster was seen in 1941 when 262 crabs were found in an oyster measuring  $85 \times 46$  mm. This oyster was in a sample dredged from Ground C already showing a high oyster mortality. Of the other live oysters in this sample, 54.6 per cent had more than 10 crabs present within the valves. Other high counts were 32, 63, 65, 81, 112, 134, 154, and 165 crabs per oyster. Multiple infestations have occurred even in small oysters, one dredged from Ground C, measuring only  $32.4 \times 14.7$  mm., containing eighteen first stage crabs.

Besides oysters containing only a single specimen of any crab stage, almost all conceivable combinations of crabs have been found among the multiply-infested oysters except those involving the larger stages.

The only other cases of multiple infestations with crabs of the genus *Pinnotheres* were reported by Ohshima (1927). As many as seven male *P. latissimus* were found within the shells of a single specimen of *Paphia*.

The crabs when present in small numbers are usually found in the mantle cavity but in the heavier infestations crabs are found throughout the water conduction system of the oyster. An extreme case is that of the multiply-infested oyster dredged in September, 1942. The mature fifth stage female crab and five first stage crabs were found on the oyster's gills, three other first stage crabs were found in the cloacal chamber and one each in the promyal and the right and left supra-branchial chambers. In one interesting case the oyster when opened disclosed a Stage V female on the gills and a Stage II female in the promyal chamber. In another case three Stage I females were found one each in the promyal and left supra-branchial chambers and one at the exit of the promyal chamber.

When only a single crab is present it is usually found on the gills (in the mantle cavity) and females of Stages III, IV, and V have been found only in this area. We may state, therefore, that this is the normal position and that aberrant positions increase with the number of the invading crabs.

Orton (1921) reported a similar state of affairs with reference to *P. pisum* in *Mytilus edulis*. Observing the feeding of these crabs through windows in the mussel's shell he noted that the larger crabs sit on the middle of the mussel with a pair of gills on each side. The smaller crabs, he reported, may be found anywhere in the mussel but generally on the gill.

In Delaware Bay, oyster crabs are found in oysters from every area sampled to date. Since this included oysters well up the bay in regions of relatively less saline waters we may state that *Pinnotheres ostreum* is able to survive wherever the oyster can. Such a statement needs some qualification, however. Invasion of oysters by a new brood of young crabs probably occurs in late summer and autumn when the haligraphic picture of the bay waters (based on extreme data for more than seven years) is almost always at or above the mean annual level of salinities in the bay (Stauber, 1943). The meager data at hand indicate that if markedly low salinities prevailed at this critical time survival of the crabs would probably be greatly lessened. Crab-infested oysters subjected to adverse environmental conditions throw

considerable light on this problem. Death of first stage crabs within the oyster has occurred as a result of over-winter storage of oysters in pits in the ground and in a home refrigerator and as a result of the subjection of oysters to periods of low salinities (spring of 1941 and late autumn of 1942 on the beds far up the bay). It is especially notable that the later stages of the female crabs were able to survive these same adverse conditions. The latter fact is clear evidence that the adaptation of the crab to life within the oyster, as is indicated by the morphological changes of the female crab, involves physiological changes as well. Since the adverse situations described above all involve persistent closure of the oyster the female crab acquires the ability (which the oyster is well known to possess) to survive periods when cessation of the food supply and interruption of a steady stream of oxygenated water for respiration occur.

Invasions of oysters by *Pinnotheres* most probably occur in the late summer and autumn. This statement is based on several types of observation. The few ovigerous females seen have been encountered only during this period. Besides, crab zoeae of various species are found during these seasons. Furthermore, the 1941 mortalities began to be noticed in September of that year. Finally, infestation of oyster spat only a few months old was observed several times. Such invasion could only have occurred in the late summer or early autumn.

The heavy crab infestations of oysters reported in Table I were extreme values. In most areas sampled fewer than twenty-five per cent of the oysters opened contained crabs of any size, Stage V females being in the great majority. In such samples multiple infestations were very unusual. This is as valid for 1941 when the general incidence was much higher as it is for 1942.

Oysters of all sizes have been found infested with crabs. All stages of crabs have been found present though no later stages of females have been seen in oysters less than one year old for reasons which are obvious. The smallest infested oyster was one less than four months old from the egg and only  $20 \times 16$  mm. in size. A second stage female crab measuring only 0.9 mm. wide was found in the mantle cavity. The left mantle was perforated and the inner right demibranch slightly eroded. These observations indicate that growth and differentiation of the crab and perceptible damage to the oyster can occur within a relatively short period of time.

The smallest oyster yet opened which has contained a mature fifth stage female was an oyster probably less than two years old and measuring only 34 mm. long. The crab had a carapace 9 mm. wide and large-crab type gill erosions were present.

#### MODE OF FEEDING

Orton's description of the feeding of *P. pisum* in *Mytilus edulis* is remarkably similar to the method of feeding of *P. ostreum* in the oyster. The larger crabs are stationed with ventral side approximated toward the inner aspect of one mantle and facing away from the oyster's gills toward the ventral margin of the shell or into the incurrent stream of water. Usually two of the oyster demibranchs project over and above the carapace of the crab and not in regular contact with the crab. The other two demibranchs are close to and at times in contact with the posterior part of the ventral surface of the crab.

The oyster feeds by straining sediment and plankton (chiefly diatoms and dino-

flagellates) from the water.<sup>4</sup> A portion of these strained particles mixed with mucus is passed anteriorly in trails along the marginal furrow of each demibranch. The palps of the oyster constitute a further sorting apparatus and some of the strained material is rejected by the palps. This rejected material is passed posteriorly and ventrally along a ciliated path on the inner aspect of the mantle to a point near the ventral edge of the shell from which it is ejected by the blowing force of the sudden closure of the oyster's valves. If the oyster crab confined its feeding activity to the constant stream of rejected material on its way from the palps it might be of some value to the oyster and could possibly be called a symbiont or at least a commensal. However, in our experience the rejected masses are too bulky to easily fit into the crab mouth and most of this material is eventually thrown off by the crab after attempting to break it up into suitable particles by the action of the mouth parts. By reaching beneath its abdomen with its claws and also by disengaging the tangled mucus-food masses caught in its legs, it obtains newly-formed, delicate, strings of food much more capable of being handled by the mouth parts and ingested. I have seen such strings completely and quickly engulfed. The crab often combs the other legs with the chelipeds to free them from entangled food masses. The mucous masses are then passed to the mouth where the mouth parts in turn gather and press the strings of food into the mouth. The fourth or last pair of walking legs are frequently worked up over the carapace of the crab to scrape forward within the easy reach of the chelipeds any food strings which may fall from the other demibranchs or even to disengage them from the marginal furrow. The large crabs range in the mantle cavity from the place where palps and gills meet to a point ventral to the adductor muscle. Under favorable conditions they are seen to be very active, almost constantly moving legs or chelipeds.

The process of feeding, however, is wasteful and much of the material brought to the mouth is handled several times before it is ingested with appreciable amounts thrown off by the mouth parts. In one case a large crab was straddling the stream of rejected mucus-food passing to the edge of the mantle from the palps yet in more than fifteen minutes' steady observation it failed even to attempt to pick it up. In the meantime the combings of the walking legs were being vigorously prosecuted.

Rathbun remarks that the food of the oyster crab is composed of the same organisms which constitute the food of the oyster plus small crustaceans not normally the food of the oyster. The complicated feeding mechanism of the oyster is chiefly one of straining particles from the water and then sorting out and discarding the larger of these particles. Much of the food passing along the marginal furrow is destined to be discarded because of particle size. It is not surprising, then, that small crustaceans (Rathbun) are included in the crab's diet in addition to the entangled smaller organisms eaten by the oyster.

Observations on the feeding of the smaller stages of *P. ostreum* (especially first and second stages) throw further light on the mechanism producing the gill damage to be described later. In maintaining its position on the demibranchs the pointed dactyli of the walking legs are regularly seen to pierce the gill tissue. In order to maintain its position several nearby plicae may be stretched out of position by the pull of the inserted dactylus. When the leg is moved the area pierced stands out as

<sup>4</sup> This description is based chiefly on the findings of Yonge (1926), Elsey (1935) and especially Nelson (1938 and personal communications).

an opaque spot on the demibranch. It appears like a condensation of tissue either by compression of surrounding parts or by invasion of leucocytes. This area is visible to the unaided eye for several hours. Some of the walking legs have been observed to scrape the gill in an attempt to engage mucous strings of food moving on the filaments (especially the principal filaments in the folds between the plicae). This scraping action may be very vigorous and may be likened to the child's use of its fingers to clean the frosting from the egg beater. Occasionally the dactylus of the scraping leg catches in a portion of the gill (ostium). The crab strains and the gill tissue stretches. When the pull becomes hard enough the leg finally completes its motion (due to the local tearing of the gill) and the affected portion of the demibranch springs back into position. The leg and cheliped would then be approximated and the combing would proceed even when no food material had been obtained by the active leg. (Since many of these observations were made in very clear water there was little material to be filtered by the oyster.) Small crabs have also actually been seen to grasp the delicate gill tissue with the cheliped and put tension on it, pulling it out of normal alignment. When released the gill was seen to be definitely injured.

In all stages of the crabs the activity was seen not to consist so much of movement from place to place but chiefly movements of legs and chelipeds related to the act of feeding.

#### INJURY TO THE OYSTER GILL AND GILL REGENERATION

Although no extensive erosions of gills were produced by crabs in the laboratory, nevertheless, the observations recorded and the activities and injuries discussed above are sufficient to account for the extensive gill damage regularly seen in crab-infested oysters.

Two general types of gill damage were noted associated with large and small crabs respectively. The large-crab type erosion (Fig. 23) shows extensive shortening in the height of one or more demibranchs over an area reaching from the anterior end of the gills where they meet the palps to a point usually ventral to the adductor muscle. The affected demibranchs may be even less than half as high as would be expected normally or as the unaffected demibranchs of the same oyster. The outer demibranchs are normally shorter than the inner ones but cases where both inners and one outer are eroded have been seen. Such specimens show the uneroded outer demibranch to be the highest but only in the region where the other gills are eroded. The edges of the eroded demibranchs are usually slightly ragged, markedly thickened, and considerably more opaque than the edges of normal oyster gills. As a result of the gill erosions as well as the more rapid growth of the mantle and shell in a ventral direction along with the growth of the crab, an enlarged mantle cavity is produced permitting ample room for the movement of the contained crab. One oyster examined demonstrated the extreme condition which could develop as a result of the presence and activity of a large crab. The oyster was 7.0 cm. long from hinge to bill and 4.8 cm. wide just anterior to the adductor muscle. The periphery of the mantle extended to the edge of the shell in all directions. The distal margin of the demibranchs, however, did not reach the ventral shell margin by 2.1 cm. at the widest part of the oyster. The left demibranchs were only 0.8 cm. high and the eroded right demibranchs only half this size. The fifth

stage female crab was 13.0 mm. wide but with outstretched legs and chelae she extended over an area 4 by 2 cm. At first glance after turning back the mantle the body of the oyster appeared to occupy only the dorsal half of the cavity formed by the two shells.

The position of the crab within the oyster was studied in relation to the orientation of the oyster on the bottom. It was found that the crab always faced away from the gill with the normal gills over the crab's carapace (Fig. 23) even when the oyster's position on the bottom made it certain that the crab had remained consistently upside down with relation to the earth's gravitational field. In the laboratory also, there were numerous cases where the crabs remained for days in an upside down position on the oyster's gills. It is this persistent maintenance of position associated with the feeding activities of the crabs which produces the type of erosions seen.

The small-crab type erosion is quite different in appearance and consists of a more local, sharply delimited erosion of one or more demibranchs (Fig. 24). Any single or contiguous demibranchs may be eroded or even all four. The earliest cases show a ragged distal edge, but further attack gradually hollows out a space in which the crab is found. The picture reminds one of a portion cut away or punched out of the edge of the gill although in two cases one of the eroded demibranchs was perforated. The margins of the damaged area are usually thickened by what appears to be accumulations of leucocytes and probably regeneration of damaged tissue. Small-crab type erosions are usually seen at the anterior ends of the demibranchs though, occasionally, typical erosions are observed at any point along the gill. The demibranchs are often eroded more than half way to the base. Very rarely in multiple infestations two areas of damaged gill are seen. Except for one case of eroded palps and several cases with a perforated or damaged mantle area in close approximation to typically eroded gills no other sites of injury to the oyster have been found. In a few cases the mantle instead of being actually perforated was blown up into a "blood blister" or a "pus pocket" as a result of the irritating activity of the crab. The localization of injury is almost conclusive proof that gill destruction is produced by the impingement of the crab's method of feeding on the oyster's food-sorting equipment. It is most likely that the initial break in the marginal furrow by causing food trails to end at the break results in intensified activity by the crab at this spot. Such gill damage is not necessary to but incidental to the mode of feeding of *Pinnotheres*. In the case of the first stage crabs, however, the exoskeleton is hard and unyielding. Certainly this contributes to the production of the early lesions though extension to the large-crab type must occur in later stages of development. Since the adaptation of the crab to the oyster has not yet reached a plane where neither suffers injury the oyster-crab, *Pinnotheres ostreum*, must be considered parasitic on the oyster. It is conceivable that further evolution by shortening the feeding time of the first stage crabs, further decreasing the strength of the exoskeleton, increasing the skill with which the crab obtains the mucus-food strings or confining its feeding to the palp-rejected masses, might result in a relationship less harmful to the oyster. The crab would then be a more efficient parasite in Swellengrebel's (1940) sense of the word.

In small-crab type erosions the gill damage frequently extends down to the base of the gill such that the suprabranchial chamber is directly visible through the most proximal portions of the water tubes. Previous types of damage described involve

chiefly a decrease in total gill surface available for feeding. The exposure of the water tubes illustrates another type of malfunction of the oyster's feeding mechanism which has been studied in more detail in the laboratory.

When the oyster's gills are experimentally damaged it can be shown that such injuries produce leaks in the water conduction system of the oyster. Since such a leak backward into the mantle cavity means that water already strained must be rehandled several times a decrease in gill efficiency results the degree of which depends on the extent of the leakage. In a typical experiment a dissecting needle was pushed into the inner left demibranch of a small oyster (No. 1) on the half shell (opened six days before) and forced distad tearing a single plica for a distance of approximately 1.8 mm. from the marginal furrow. When the surrounding plicae were tested with ink suspension<sup>5</sup> some of the latter was seen to escape from the torn plica. A portion of the water being strained by six adjacent plicae was observed to be leaking back into the mantle cavity.

When more extensive V-shaped areas were cut out of a demibranch by scissors the leakage was much greater in volume and water strained from a much larger area was involved. A market-size oyster (No. 2) opened and studied on the half shell will serve as a typical example. Such preparations live as long as six weeks in aquaria, especially if water temperatures are generally below 20° C. A wedge of the outer left demibranch was cut out which measured about 1 cm. across the widest part (along the marginal furrow). At the apex the cut penetrated to a point slightly more than halfway to the gill axis or base. The excision was located just posterior to the anterior third of the demibranch. When ink suspension was placed on this demibranch anterior to the cut practically none escaped in the normal fashion from the cloacal chamber but streamed forcefully out of the openings of the severed water tubes. In consequence, the water strained by more than a third of one demibranch had to be rehandled. Ink placed on the inner left demibranch at the level of the cut on the outer left demibranch also escaped in large part from the cut water tubes of the outer left demibranch. Such a loss of efficiency of parts of two gill leaves must reduce the amount of food an oyster can obtain.

As a result of these findings newly-opened, crab-infested, gill-eroded oysters were studied to see whether such leakages occurred naturally. For reasons to be discussed later; namely, the rapid rate of healing and regeneration of the oyster, good examples were hard to find. One notable case, however, examined in January, clearly demonstrated the existence of such injuries. Water tubes of the two inner demibranchs were exposed in the central portion of a rather extensive small-crab type erosion. When tested on the half shell with ink, both affected demibranchs showed significant leaks. Not only the usual loss from ink placed anteriorly to the lesion but even some placed posteriorly escaped from the damaged area. The latter indicates that some water was moving *anteriorly* in the supra-branchial chamber above the inner right demibranch for a short distance.

<sup>5</sup> The ink suspension is made by diluting Higgins' Eternal Black Carbon ink with bay water. A dilution of about 1-10 with some subsequent evaporation makes a suspension suitable for use. Placed on the gill of a normal oyster the particle sizes are generally so small that almost all the ink is swept through the ostia into the water tubes. In the intact oyster it appears as a cone-shaped black cloud issuing from either or both of the exhalent chambers on the dorsal side of the oyster.

In another experiment a small oyster on the half shell was placed in a dish with four second stage female crabs. Three days later parts of the demibranchs showed twisted and fused plicae (healed), broken plicae and irregularities of form of filaments not normally seen. One particular area showed extensive damage of nine plicae including destruction of the marginal furrow of eight of these.

To explain the fact that serious leaks are not often naturally found we studied the daily changes in experimentally produced gill lesions of oysters on the half shell. The lesions healed in such a remarkably short period of time that we can easily understand our previous difficulties.

In oyster No. 1, for example, twenty-four hours after the gill injury was produced marked healing had occurred. Three days later (water temperatures in intervening period below 10° C.) much of the damage had been repaired. Anatomically, as well as by ink test, the hole had been plugged by tissue though reorganization of filaments and the marginal furrow had not occurred.

Another small oyster (No. 3) healed much more rapidly. In this case ink escaped from three small experimentally injured areas when tested soon after injury. Less than 24 hours later the holes were sufficiently bridged over by local contractions and regeneration to make water tight plugs.

Another small oyster receiving wedge-shaped excisions of both left demibranchs healed over the exposed water tubes of the shallower one and more than half of the more seriously damaged one in less than a day. Total closure of the wound was accomplished by the fourth day.

Oyster No. 2 likewise healed over though here the excision was so broad and deep that a longer period was necessary for recovery. At the twenty-four interval little microscopically-visible healing could be seen but loss of ink occurred only when placed over a space extending twelve plicae anterior to the excised area. A large amount of ink placed on the inner left demibranch at the level of the injury still showed some loss from the exposed water tubes of the outer left gill leaf.

By the fourth day almost half the exposed openings were bridged over with tissue. On the sixth day ink placed more than two plicae anterior to the edge of the excision no longer escaped from the cut water tubes. When placed proximal to the cut, ink escaped from less than half the originally exposed water tubes and then only at the apex of the wedge where the most exposure occurred. Now, also, only a small amount of the ink placed on the other demibranch (inner left) was lost at the cut surface. By the tenth day microscopic examination and ink tests showed that the gap had been completely closed. Although the damage was not yet all repaired the most serious effects were circumvented. It is reasonable to suspect that the intact oyster under more favorable conditions could respond even more quickly than has been demonstrated in these experiments. Since the crab probably does not confine its activity to a single plica for any considerable length of time a reasonable explanation of the appearance of the gill erosions in recently-opened oysters is at hand.

Gill-damaged oysters lose their crabs at times or eject dead ones. Eventual reconstitution of essential gill structure may then take place though cicatrization and "scar" formation are noticeable. None of the interesting histological aspects of the healing process have yet been studied.

## OTHER EFFECTS OF INVASION

Oysters exposed to prolonged storage in air or earth were found to vary in their keeping qualities. It was soon noted that most of the weaker oysters were crab-infested. Whether this was due to the poorer condition of the oyster, irritation by the crab or greater loss of shell liquor due to the enlarged mantle cavity is not known. From the commercial viewpoint, however, this is a serious matter. Much of these losses automatically are circumvented because the oysterman selects for market only his best oysters. It should be emphasized here that even in multiple infestations the presence of more than one gill erosion is extremely rare. An oyster containing fifty crabs is more likely to have no greater eroded area than an oyster containing only a single crab. It is our belief, therefore, that multiple infestations are the result not only of the presence of large numbers of invasive crabs in the water but also of the weakness produced by the activity of a primary crab invasion or any other condition producing weak oysters. The malfunction or even complete stoppage of the feeding mechanism under such conditions may account for the lack of further gill erosions. Such oysters were probably unable to survive the winter and contributed to the heavy mortalities which occurred in some areas after the winter of 1941-1942.

## INVASION OF OYSTERS BY CRABS

Orton believes that the hard, flat shell of the invasive first stage crab (*P. pisum*) is of value not only in slipping within the valves of the mollusc but in avoiding crushing by the closing valves. We believe this to be largely true for *P. ostreum* and the oyster, though successful invasion does not always occur.

In one experiment an oyster dredged for market was placed in an aquarium with one hundred and fifty first stage *Pinnotheres*. The crabs were scattered over the dish at the start of the trial. Within a short time many were seen lined up around the oyster from hinge to bill mostly at the places where the two valves come together. Seven days after the start only twenty-five live and twenty dead crabs were found in the dish but none were now present on the oyster. The oyster was then opened. There were forty-nine live crabs in the left valve on opening, most of these were around the edges of the shell on the incumbent side where the oyster had apparently begun to wall off the crabs. Twenty-six more crabs were found on the ventral side of the oyster, mostly on the gills but no gill erosions were present. Sixteen crabs were on the dorsal side, three in the promyal chamber, two deep in the cloacal chamber, three deep in the left suprabranchial, three in the right suprabranchial chamber and two so deep in the water tubes of the anterior end of the inner right demibranch that the gill had to be dissected to free them. The oyster was 108 × 66 mm. in size and contained one hundred and four of the crabs. All but one of the original crabs were found. Two of the other trials with various oysters yielded invasions of seventy-seven of one hundred and eighty-one and forty-nine of one hundred and twenty-nine crabs.

In all trials a few dead crabs were seen and in one series of closely examined oysters exposed to only a few crabs the fate of several was followed. A typical case showed a crab attached to the oyster shell opposite the opening of the cloacal chamber. It had assumed this position within a half hour and almost appeared as

though its posterior end were caught within the oyster's shell. Seven hours later it had scarcely moved in position but one hour later it was found on the bottom of the dish on its back, dead, directly below the position it had been occupying on the shell of the oyster. It had apparently attempted entry and partially succeeded but was compressed until dead and then ejected. In another case a weak crab was removed from the bottom of a dish for examination. It had lost parts of two legs on one side. In still another instance two specimens which had effected entry, so far as could be detected, were later ejected from the oyster as dead crabs. These probably had effected almost but not complete entry.

#### NOTE ON COLLECTING SPECIMENS FOR FUTURE STUDY

In commercial packing of oysters the oysters are drained—in part to insure accurate measurement of the volume of the oysters opened. This volume is the basis of paying the shucker or opener. The oysters are later washed and drained again before shipment. Catching the first drainings in pails and then placing the material collected in shallow pans or dishes of bay water allows the crabs, freed in the opening of the oysters, to be readily recognized and collected.<sup>6</sup> We have been successful in obtaining specimens of all stages of *P. ostreum* in this fashion especially from areas where crab density is very low.

#### DISCUSSION

It is obvious from the data presented that *Pinnotheres ostreum* can no longer be considered other than a parasite of the oyster. That we witnessed a most unusual sequence of events in 1941 is without question for the dramatic appearance of an oyster containing one hundred or more crabs can hardly be overstated. The subsequent decrease of numbers in 1942 is another example of the population cycles so often seen in nature. We have also seen the abrupt rise and fall in numbers of starfish, *Stylochus ellipticus* and *Polydora ligni* (Nelson and Stauber, 1940), on the oyster grounds in Delaware Bay in recent years. The rise of each was associated with heavy, localized mortalities.

It is not surprising that the evidence gathered here was not previously noted. The small size of the first stage crabs and the need for careful opening and search of oysters in order to detect their presence are prime reasons. For example, we have repeatedly seen cases with gill erosions but could detect no crab on the first examination. Placing these oysters on the half shell in dishes of bay water for a few hours a subsequent examination would often reveal the presence of a crab. Furthermore, among oysters held out of water for a time we have found specimens of first stage *P. ostreum* dried fast to the edge of the oyster's shell as though they had attempted to leave the unfavorable environment and were trapped by lack of an ambient fluid medium in which to move. This being the case it would take such an unprecedented increase in density as occurred in 1941 to focus one's attention on the causal relationship between crab and oyster gill damage. The very fact that in over one hundred years only two specimens of male crabs were obtained by collectors is in itself suggestive. Nor can the fact that oystermen always select for quality of meats be overlooked. Only someone searching for and among poor oysters or investigating causes of mortality would be apt to find the crabs.

<sup>6</sup> I am indebted to Dr. T. C. Nelson for this fruitful suggestion.

The large crabs, however, have long been known and their very existence means that the earlier stages were also present though undetected. Dean's description of malformed or stunted palps with thickened outgrowths is clearly indicative that the parasitism has not been acquired recently. But the shortening of demibranchs on the affected side of an oyster containing a Stage V crab is not always readily distinguished especially since there are minor differences in the heights of the demibranchs normally. In a significant proportion of the cases also, since the erosions are present on only one or two of the demibranchs, the damaged area is shielded from cursory glance by the unaffected gills which cover them. This is even true of the small-crab type lesion in many cases.

It would be of great interest to learn whether these findings are duplicated at other points along the eastern seaboard where the presence of *Pinnotheres ostreum* within the valves of *Gryphaca virginica* has long been known to exist.

#### SUMMARY

1. All the parasitic stages of *Pinnotheres ostreum* are described, many for the first time. Besides the invasive first stage male and female crabs, one other male stage and four other female stages are reported.

2. The first crab stage (male or female) is hard-shelled, relatively flat and hairy with a distinctive brown color and white markings.

3. Subsequent stages are quite different with membranaceous, yielding carapace, slender legs, and more rounded body form.

4. The later stages of the females are distinguished from one another by size, color, width and shape of abdomen with relation to the rest of the body and size and differentiation of the pleopods. Maturity is usually reached in the fifth stage female and ovigerous crabs have been seen.

5. All stages of crabs have been found in oysters either singly or in various combinations except that no more than one specimen of a Stage III, IV, or V female has ever been observed within a single oyster.

6. Multiple infestations have been observed which involve chiefly first stage crabs. One oyster contained 262 of these crabs.

7. The oyster crab feeds chiefly on the particulate food material strained from the water by the oyster's food sorting mechanism.

8. In so feeding the crab injures the demibranchs of the oyster causing erosions which interfere with normal feeding of the oyster. These erosions are sharply localized in oysters with first stage crabs.

9. Damage to the oyster gills involves a reduction in the amount of area available for food collecting and in the efficiency of both the straining and the collecting mechanisms. The more serious lesions may also show leakages of the water pumping or conduction system which is such an important feature of food sorting.

10. Rapid healing and regeneration of gill tissue almost keeps pace with destruction and probably saves many oysters from death.

11. The hard shelled, almost tick-like, invasive first stage crabs are less adapted to life within the oyster than the modified later stages. The latter are more resistant to their host's exposure to unfavorable environmental conditions such as storage out of water.

12. Experimental invasion of oysters by crabs has been accomplished but artificial duplication of typical, extensive gill lesions has not yet been obtained.

13. The unprecedented invasion of oysters in 1941 was followed by a lesser invasion in 1942 and probably will follow the usual course of population cycles in future years.

14. The parasitic nature of the crab's relations with the oyster is outlined and reasons suggested why this previously was not found out.

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# THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

## THE MARINE BIOLOGICAL LABORATORY

FORTY-SEVENTH REPORT, FOR THE YEAR 1944—FIFTY-SEVENTH YEAR

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## II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such

case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

*Now, therefore*, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

*Witness* my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,  
*Secretary of the Commonwealth.*

### III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Massachusetts, at 11:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Mass., at 10 A.M. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years; and in addition there shall be two groups of Trustees as follows:

(B) Trustees *ex officio*, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk;

(C) Trustees Emeritus, who shall be elected from the Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. Any person interested in the Laboratory may be elected by the Trustees to a group to be known as Associates of the Marine Biological Laboratory.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

---

## IV. THE REPORT OF THE TREASURER

### TO THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY:

*Gentlemen:*

Herewith is my report as Treasurer of the Marine Biological Laboratory for the year 1944.

The accounts have been audited by Messrs. Seamans, Stetson, and Tuttle, certified public accountants. A copy of their report is on file at the Laboratory and inspection of it by members of the Corporation will be welcomed.

The principal summaries of their report—The Balance Sheet, Statement of Income and Expense, and Current Surplus Account—are appended hereto as Exhibits A, B and C.

The following are some general statements and observations based on the detailed reports:

#### *I. Assets*

##### *1. Endowment Assets*

As of December 31, 1944, the total book value of all the Endowment Assets, including the Scholarship Funds, was \$983,900.57, a loss for the year of \$19,808.06.

The Scholarship Funds were increased by the gift of \$5,000.00 from Bishop James E. Cassidy of Fall River to establish the "Reverend Arsenious Boyer Burse." The principal losses incurred were due, as in the previous year, to the foreclosure of mortgage participations on New York City realty and the subsequent sale of the properties. In 1944 a four story tenement at 4856 Broadway on which the Laboratory held a mortgage investment of \$30,057.10 was sold, the Laboratory receiving \$6,026.00 cash and a new mortgage participation for \$17,000, and sustaining a loss of \$7,031.10. The property at 47 Murray Street, a five story loft building, was sold entirely for cash, at a loss of \$9,569.24 on an investment of \$21,928.75.

At the end of the year \$803,403.76 was invested in marketable securities (bonds, preferred stocks and common stocks) with a market value of \$825,005.80. \$163,769.79 was invested in mortgage participations on New York City real estate and in real estate participations resulting from mortgage foreclosures. \$16,727.02 was in uninvested principal cash.

The Treasurer's estimate of the actual value of the \$163,769.79 in mortgage and real estate participations held on December 31 is \$87,750.00. With the market value of \$825,005.80 on marketable securities and the \$16,727.02 in cash this makes a total current valuation of \$929,482.82 compared with total book value of \$983,900.57 and original capital value of \$1,116,924.25.

## *2. Plant Assets*

The total of Plant Assets (excluding the Gansett and Devil's Lane tracts) was \$1,333,726.48 after deduction of \$656,341.78 accumulated Depreciation Reserve, a decrease for the year of \$7,699.40. Depreciation charges for 1944 were \$26,929.31. The Reserve Fund was increased to a total of \$16,895.62 by \$3,529.41 transferred from current income (representing \$279.41 profit on sale of Gansett lots, the Crane Company dividends, and part of the dividends on the General Biological Supply House stocks) and \$93.99 interest received from the temporary investment of \$10,000 of the Reserve Fund in U.S.A. Treasury bonds.

## *3. Current Assets*

Current Assets including cash, inventories, and investments not in the Endowment Funds at cost, amounted to \$202,239.67, an increase of \$8,127.98. Current Liabilities totalled \$2,181.09. The special reserve fund for repairs and replacements, made up of a portion of the 1943 income from the United States Navy rentals, and the value of certain equipment received from the Navy in lieu of restoration and repairs upon termination of the Navy lease, was \$15,998.62 at the end of the year. Current Surplus was \$184,059.96, \$4,442.14 under the total for 1943.

## *II. Income and Expenditures*

Total Income was \$164,240.13, an increase of \$4,943.19 over the 1943 income. Total Expenses were higher, \$160,013.13, including Depreciation Reserves of \$26,929.31 and special hurricane damage repairs of \$2,466.17, but there was an actual net surplus of \$4,227.00 for the year.

This surplus compares favorably with the \$19,323.67 surplus in 1943 which resulted largely from the \$20,150.00 rental from the United States Navy combined with reduced expenditures, and the deficit of \$17,211.93 for 1942. Some of the reductions in 1944 income were a decline of \$3,600.91 in endowment income, a

loss of \$6,000 in net income from the Supply Department compared with 1943, and a reduction of \$2,286.00 in the dividends from the General Biological Supply House. The principal gains were Mrs. W. Murray Crane's gift of Otis Elevator stock valued at \$2,325.00, and an increase of over \$4,000 in net income from Research.

The income and expense items, although more normal than in 1943, still do not reflect what may be regarded as regular operations. Expenditures for equipment and necessary improvements, for example, are still unavoidably under what they should be to maintain the Laboratory at full efficiency. Some reserves have been built up for a few of these expenditures, but the Laboratory needs a larger endowment income to take care of maintenance.

## EXHIBIT A

## MARINE BIOLOGICAL LABORATORY BALANCE SHEET, DECEMBER 31, 1944

*Assets*

## Endowment Assets and Equities:

|  |               |                      |
|--|---------------|----------------------|
| Securities and Cash in Hands of Central Hanover Bank and Trust Company, New York, Trustee..... | \$ 968,737.59 |                      |
| Securities and Cash in Minor Funds.....  | 15,162.98     |                      |
|  |               | <u>\$ 983,900.57</u> |

## Plant Assets:

|  |                       |                       |
|--|-----------------------|-----------------------|
| Land.....                              | \$ 111,425.38         |                       |
| Buildings.....                         | 1,327,675.21          |                       |
| Equipment.....                         | 186,122.42            |                       |
| Library.....                           | 329,639.23            |                       |
|  | <u>\$1,954,862.24</u> |                       |
| Less Reserve for Depreciation.....     | 656,341.78            | \$1,298,520.46        |
| Reserve Fund, Securities and Cash..... |                       | 16,895.62             |
| Book Fund, Securities and Cash.....    |                       | 18,310.40             |
|  |                       | <u>\$1,333,726.48</u> |

## Current Assets:

|  |              |                       |
|--|--------------|-----------------------|
| Cash.....  | \$ 27,513.52 |                       |
| Accounts Receivable.....                           | 12,357.71    |                       |
| Inventories:                                       |              |                       |
| Supply Department.....                             | \$ 43,964.75 |                       |
| Biological Bulletin.....                           | 19,498.15    | 63,462.90             |
| Investments:                                       |              |                       |
| Devil's Lane Property.....                         | \$ 46,260.84 |                       |
| Gansett Property.....                              | 1,900.42     |                       |
| Stock in General Biological Supply House, Inc..... | 12,700.00    |                       |
| Other Investment Stocks.....                       | 20,095.00    |                       |
| Retirement Fund.....                               | 12,966.30    | 93,922.56             |
| Prepaid Insurance.....                             |              | 4,184.40              |
| Items in Suspense.....                             |              | 798.58                |
|  |              | <u>\$ 202,239.67</u>  |
| Total Assets.....                                  |              | <u>\$2,519,866.72</u> |

## MARINE BIOLOGICAL LABORATORY

*Liabilities*

## Endowment Funds:

|  |               |
|--|---------------|
| Endowment Funds . . . . .                          | \$ 967,113.46 |
| Reserve for Amortization of Bond Premiums. . . . . | 1,624.13      |

|                       |               |
|-----------------------|---------------|
|                       | \$ 968,737.59 |
| Minor Funds . . . . . | 15,162.98     |

\$ 983,900.57

## Plant Funds:

|  |                |
|--|----------------|
| Donations and Gifts . . . . .                                    | \$1,172,564.04 |
| Other Investments in Plant from Gifts and Current Funds. . . . . | 161,162.44     |

\$1,333,726.48

## Current Liabilities and Surplus:

|  |             |
|--|-------------|
| Accounts Payable . . . . .                     | \$ 2,181.09 |
| Reserve for Repairs and Replacements . . . . . | 15,998.62   |
| Current Surplus (Exhibit C) . . . . .          | 184,059.96  |

\$ 202,239.67

Total Liabilities . . . . . \$2,519,866.72

## EXHIBIT B

MARINE BIOLOGICAL LABORATORY INCOME AND EXPENSE,  
YEAR ENDED DECEMBER 31, 1944

|   | Expense     | Total<br>Income | Expense     | Net<br>Income |
|---|-------------|-----------------|-------------|---------------|
| Income:   |             |                 |             |               |
| General Endowment Fund . . . . .  |             | \$ 27,291.16    |             | \$ 27,291.16  |
| Library Fund . . . . .  |             | 5,718.91        |             | 5,718.91      |
| Donations . . . . .   |             | 2,325.00        |             | 2,325.00      |
| Instruction . . . . .   | \$ 8,485.47 | 5,570.00        | \$ 2,915.47 |               |
| Research . . . . .  | 4,981.44    | 13,654.38       |             | 8,672.94      |
| Evening Lectures . . . . .  | 45.85       |                 | 45.85       |               |
| Biological Bulletin and Membership Dues . . . . .                       | 4,102.15    | 7,952.96        |             | 3,850.81      |
| Supply Department . . . . .   | 37,307.27   | 45,588.92       |             | 8,281.65      |
| Mess . . . . .  | 20,225.08   | 17,878.72       | 2,346.36    |               |
| Dormitories . . . . .   | 30,761.47   | 13,190.82       | 17,570.65   |               |
| (Interest and Depreciation charged to<br>above 3 Departments) . . . . . | 25,076.43   |                 |             | 25,076.43     |
| Dividends, General Biological Supply<br>House, Inc. . . . .             |             | 16,510.00       |             | 16,510.00     |
| Dividends, Other Investment Stocks . . . . .                            |             | 785.00          |             | 785.00        |
| Rents:  |             |                 |             |               |
| Bar Neck Property . . . . .   | 759.46      | 4,800.00        |             | 4,040.54      |
| Janitor House . . . . .   | 21.35       | 360.00          |             | 338.65        |
| Danchakoff Cottages . . . . .   | 278.44      | 643.33          |             | 364.89        |
| Rooms in Laboratory, Special . . . . .                                  |             | 420.00          |             | 420.00        |
| Sale of Library Duplicates and Micro Film . . . . .                     |             | 194.90          |             | 194.90        |
| Microscope and Apparatus Rental . . . . .                               |             | 1,168.24        |             | 1,168.24      |
| Sundry Income . . . . .   |             | 187.79          |             | 187.79        |

# REPORT OF THE TREASURER

9

## Maintenance of Plant:

|                                     |           |           |
|-------------------------------------|-----------|-----------|
| Buildings and Grounds.....          | 18,759.11 | 18,759.11 |
| Apparatus Department.....           | 3,765.15  | 3,765.15  |
| Chemical Department.....            | 1,681.50  | 1,681.50  |
| Library Expense.....                | 6,756.08  | 6,756.08  |
| Workmen's Compensation Insurance... | 440.09    | 440.09    |
| Truck Expense.....                  | 327.35    | 327.35    |
| Bay Shore Property.....             | 93.41     | 93.41     |
| Great Cedar Swamp.....              | 20.25     | 20.25     |

## General Expenses:

|   |                     |                     |
|---|---------------------|---------------------|
| Administration Expense.....                                   | 15,275.38           | 15,275.38           |
| Endowment Fund Trustee and Safe-Keeping.....                  | 1,015.28            | 1,015.28            |
| Bad Debts.....  | 592.50              | 592.50              |
| Special Repairs on account of 1944 Hurricane Damage.....      | 2,466.17            | 2,466.17            |
| Reserve for Depreciation.....                                 | 26,929.31           | 26,929.31           |
|   | <u>\$160,013.13</u> | <u>\$164,240.13</u> |
|   |                     | <u>\$100,999.91</u> |
| Excess of Income over Expense carried to Current Surplus..... | 4,227.00            | 4,227.00            |
|   | <u>\$164,240.13</u> | <u>\$105,226.91</u> |

## EXHIBIT C

### MARINE BIOLOGICAL LABORATORY, CURRENT SURPLUS ACCOUNT, YEAR ENDED DECEMBER 31, 1944

Balance January 1, 1944..... \$188,502.10

#### Add:

|  |             |                     |
|--|-------------|---------------------|
| Excess of Income over Expense.....                   | \$ 4,227.00 |                     |
| Gain on Gansett Lots Sold.....                       | 176.04      |                     |
| Bad Debts Recovered.....                             | 37.64       |                     |
| Reserve for Depreciation charged to Plant Funds..... | 26,929.31   | 31,369.99           |
|  |             | <u>\$219,872.09</u> |

#### Deduct:

#### Payments from Current Funds during Year for Plant

##### Assets:

|                |             |
|----------------|-------------|
| Buildings..... | \$ 3,064.00 |
| Equipment..... | 1,542.52    |
| Library.....   | 5,559.12    |

\$10,165.64

Less Received for Plant Assets Sold..... 172.00

\$ 9,993.64

Pensions Paid..... \$ 3,460.00

#### Less:

|  |          |        |
|--|----------|--------|
| Retirement Fund Income.....                            | \$223.07 |        |
| Retirement Fund Gain on Securities.....                | 351.86   |        |
| Retirement Fund, Recovery on account of 1943 loss..... | .51      | 575.44 |

\$ 2,884.56

## MARINE BIOLOGICAL LABORATORY

## Transfers to Reserve Fund:

|   |             |
|---|-------------|
| Portion of Dividends from General Biological Supply House, Inc..... | \$ 2,500.00 |
| Dividends from Crane Company.....                                   | 750.00      |
| Profit on Gansett Lots for 1943.....                                | 279.41      |

---

\$ 3,529.41

|   |             |
|---|-------------|
| Building Fixtures and Equipment Received from First Naval District, transferred to Plant Funds. | \$ 7,225.00 |
| Less Loss on Fixtures and Equipment Discarded   | 620.48      |

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\$ 6,604.52

|   |           |
|---|-----------|
| Repairs and Replacements Made by First Naval District during their occupancy of properties, set up as a Reserve | 12,800.00 |
|---|-----------|

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35,812.13

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Balance, December 31, 1944..... \$184,059.96

Respectfully submitted,  
DONALD M. BRODIE,  
*Treasurer*

## V. REPORT OF THE LIBRARIAN

The sum of \$11,239.77 appropriated to the library in 1944 was expended as follows: books, \$760.49; serials, \$2,626.99; binding, \$884.00; express, \$60.14; supplies, \$416.17; salaries, \$6,239.77; back sets, \$214.50; insurance, \$50.00; sundries, \$2.21; total, \$11,254.27. The cash earnings of the library reverting to the laboratory were \$194.90: from sale of duplicates, \$38.73; microfilms, \$144.86; serials lists, \$11.31.

Of the Carnegie Corporation of New York Fund, \$2,433.69 was expended for the completion of five and partial completion of nine back sets and two books.

The sum appropriated by the Woods Hole Oceanographic Institution for 1944 was \$1,900.00. A balance of \$263.08 remaining from 1943 made an available total of \$2,163.08. Of this sum \$113.69 was expended on current books and journals and \$1,100.00 on salaries, leaving a balance of \$949.39. A comparison of the amount spent on current books, journals and back sets during the pre-war years with that of the war years will show that this accumulating budget balance will be expended when the material for which it was designated shall have become available.

During 1944 the library received 678 current journals: 248 (10 new) by subscription to the Marine Biological Laboratory; 15 (none new) to the Woods Hole Oceanographic Institution; exchanges 201 (three new) with the "Biological Bulletin" and 23 (one new) with the Woods Hole Oceanographic Institution publications; 186 as gifts to the former and five to the latter. The Marine Biological Laboratory acquired 169 books: 119 by purchase of the Marine Biological Laboratory; six by purchase of the Woods Hole Oceanographic Institution; nine gifts from the authors, 22 from the publishers and 13 from miscellaneous donors. There were 18 back sets of serial publications completed: ten purchased by the Marine Biological Laboratory (five with the "Carnegie Fund"); two secured by exchange with the "Biological Bulletin"; one by exchange with the Woods Hole Oceanographic Institution publications; and five by duplicate material exchange and by

gift. Partially completed sets were 59: purchased by the Marine Biological Laboratory, 23 (nine with "Carnegie Fund"); by exchange with the "Biological Bulletin," one; and by exchange of duplicate material and by gift, 35. In addition, 15 of the odd journal numbers presented by Dr. Dorothy R. Stewart (126 in all) were fitted into gaps in our sets.

The reprint additions to the library number 2,404: current of 1943, 401; current of 1944, 58; and of previous dates, 1,945. A total of 3,957 reprints, 1,321 not duplicates of our holdings, were presented to the library: 1,378 by Mrs. G. N. Calkins; 2,306 by Dr. Dorothy R. Stewart; 192 by Dr. Libbie H. Hyman; and 81 by Dr. D. A. Fraser.

It is with great pleasure that two very valuable gifts are acknowledged as presented to the library this year. Dr. Walter E. Garrey has presented his collection of reprints to be incorporated in the library's reprint holdings. As yet no count of these has been made. More detailed acknowledgment will occur in a later report. The same delayed account will be given of the reprints from Dr. E. B. Meigs' library, a gift of Mrs. Meigs. In addition to the reprints, Mrs. Meigs included in her gift long runs of fourteen different journals. As a further gift from Mrs. Meigs three of these sets will be bound and, with an appropriate book plate inserted, will be substituted for the old volumes now in the library.

At the end of the year 1944 the library contained 52,885 bound volumes and 133,054 reprints.

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## VI. THE REPORT OF THE DIRECTOR

### TO THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY:

*Gentlemen:*

I beg to submit the following report of the fifty-seventh session of the Marine Biological Laboratory for the year 1944.

During the year the Laboratory has coped with difficulties brought on by the war—shortage of labor, and materials much needed for research and for the maintenance of the plant and the Supply Department—and with a hurricane which fortunately did not seriously damage our buildings. The immediate problem now reflects the encouraging change in the general situation throughout the country; it is to find laboratory space for the large number of investigators who expect to return in 1945.

#### 1. Attendance

The anticipated increase in the number of investigators and students indicates that we have already passed the low point in the curve of attendance. This can be seen in the chart on page 20 which forms a part of the report prepared for the Committee of Review. Our numbers in the five year period from 1936-1940 were the greatest in the history of the Laboratory; in 1942 they decreased by nearly 50 per cent. The year 1943 showed a still further decline. A definite improvement is seen in the record of 1944, as shown in the Tabular View of Attendance on page 21. The prospects for a still larger number in 1945 are excellent.

From the curves on the chart, one can see the effect on attendance of changing conditions in the Laboratory and in the country at large. When new buildings

were erected here, the attendance increased sharply. The first World War reversed the upward trend, but only for one year. The business depression, felt from 1932-1935, affected chiefly the number of "New Investigators." The present war has reduced our attendance to the level of 25 years ago. After 1945 there should be a rapid rise, but with our present buildings and equipment we cannot accommodate more investigators and students than we had in 1940.

New Investigators are those who come here for the first time; after the first year they are classified among "Returning Investigators." They are chiefly "Investigators under Instruction," that is, graduate students and Fellows. Over a long period of years they have constituted nearly one third of all investigators in attendance. Recently this proportion, as shown in the Tabular View of Attendance, has grown smaller; but actually, until the war, the number of graduate students, Fellows, and young instructors present each summer did not diminish. What happened was that many came as Research Assistants, probably for economic reasons.

Since 1941 the number of beginning investigators has declined by about 75 per cent from its previous level. The loss of so large a proportion of this important source of new members and future supporters of the Laboratory will be felt for many years. We fervently hope that those who are now prevented from working here will eventually return to us.

It is a pleasure to report that the number of institutions represented during the war years has not greatly diminished, and that the list of supporting institutions receives new additions every season.

## *2. Laboratory Activities*

During the summer all of the usual activities of the Laboratory were carried on. After a lapse of one year, the weekly seminars were resumed, nine being held. In addition to these, several small groups of investigators met to discuss topics in which all were especially interested. It was the general opinion that more meetings of this kind should be held. All of the courses of instruction were given, the total registration of students being 75, a moderate increase over the preceding year. Dr. John B. Buck, who served for two seasons with signal success as head of the Invertebrate Zoology course, resigned at the end of the summer. The Committee on Instruction accepted his resignation with regret, and selected Dr. Frank A. Brown, of Northwestern University, to succeed him as instructor in charge.

## *3. Associates*

The Trustees, at the regular meeting this year, directed the Executive Committee to appoint a committee to consider the advisability of establishing a new kind of membership in the Corporation, to which those interested in the welfare of the Laboratory might be elected. Mrs. W. Murray Crane, Mr. Lawrence Saunders, Dr. J. P. Warhase (all of whom were elected to membership in the Corporation at the August meeting), Dr. G. H. A. Clowes, and the Director, were asked to discuss the matter. This committee felt that there are many people without special training in Biology, who have a sincere interest in the Laboratory, and that such friends would appreciate a formal connection with it. Following the Committee's approval

of the plan, a special meeting of the Trustees was held in New York on December 9, 1944 to amend the By-laws so that this new type of membership could be made possible. The amendment which was adopted reads as follows: "Any person interested in the Laboratory may be elected by the Trustees to a group known as 'Associates of the Marine Biological Laboratory.'" It is hoped that both summer and permanent residents of Woods Hole and the vicinity may become members, and that friends in other parts of the country may also join.

At this special meeting the opinion was voiced by several Trustees that a winter meeting should be held regularly in order that current Laboratory problems could be discussed.

#### 4. *The Committee of Review*

When the Friendship Fund in 1924 contributed a large sum to the Laboratory for endowment purposes, the Trustee of the endowment was directed to call once every ten years upon a Committee of Review to make a study of the work of the Laboratory. This Committee, which consists of nine members, includes a representative of the National Academy of Sciences, of the National Research Council, of the American Association for the Advancement of Science, and one professor of Biology from each of the following universities: Chicago, Columbia, Harvard, Pennsylvania, Princeton, and Yale. Its function is to determine whether the Laboratory continues to perform valuable services in biological research. The complete text of the Deed of Trust, in which the duties of the Committee are set forth, is printed in the 26th Annual Report which appears in Vol. 47 of the "Biological Bulletin."

The Committee first met in 1934 and voted that the Laboratory was satisfactorily fulfilling the purpose for which the endowment was given. The second decennial Committee met this year. Its findings, and the statement of the President and Director of the Laboratory regarding our activities during the years 1934-1943 are appended to this report. The Committee, in addition to its formal vote of approval, pointed out that in order to maintain a high level of usefulness, the Laboratory should secure additional funds for endowment and for purposes which are specified in their report. These recommendations, coming from a group of biologists, the majority of whom were not connected with the administration of the Laboratory, should be given most careful consideration.

#### 5. *The Hurricane*

The September hurricane did not seriously damage our buildings. No water came in, as happened in the 1938 storm, but roofs and windows suffered. Some of the slate from the Dormitory and Apartment House roofs was blown off, many pieces imbedding themselves in distant houses. Fortunately no one was struck by these flying missiles. The Cayadetta wharf was practically demolished, and the sea wall badly broken by the tremendous waves that tossed great stones on to the street. The wharf has been partially restored by the Oceanographic Institution which has used it for the past two years. Had the full fury of the storm struck at high tide we might well have sustained a loss, due to sea water, even greater than that which we suffered in 1938. The wind, whose velocity far exceeded that

of the previous hurricane, levelled a great number of trees in the Gansett and Devil's Lane tracts, on Dr. Clowes' property, and in the Fay Woods.

Against the destructive power of winds we can do little, but it is possible to protect the Brick Building from high water. The matter of increased protection should be given consideration.

#### 6. *Loss by Death*

This year the Corporation has lost by death Prof. William Trelease who was elected in 1888 at the first regular meeting of the Trustees after the incorporation of the Laboratory.

#### 7. *Gift*

The Laboratory acknowledges with sincere appreciation the receipt of 100 shares of Otis Elevator stock valued at \$2,325.00, a gift of Mrs. W. Murray Crane.

#### 8. *Election of Trustee*

At the meeting of the Corporation held August 8, 1944, L. G. Barth, Associate Professor of Zoology at Columbia University, was elected to fill the vacancy caused by the resignation of Prof. I. F. Lewis.

#### 9. There are appended as parts of this report:

1. Memorial to Dr. Caswell Grave.
2. The Report of the Committee of Review.
3. The Decennial Review—Submitted to the Committee of Review.
4. The Staff.
5. Investigators and Students.
6. Tabular View of Attendance, 1940-1944.
7. Subscribing and Co-operating Institutions.
8. Evening Lectures.
9. Shorter Scientific Papers.
10. Members of the Corporation.

Respectfully submitted,  
CHARLES PACKARD,  
*Director*

#### 1. MEMORIAL TO DR. CASWELL GRAVE

*By Prof. R. A. Budington*

It is with the greatest reluctance, and with true sorrow, that today we must include among those permanently lost to the Corporation the name of Caswell Grave. Those who knew him, as most of us here did, will miss his genial personality, with his habit of industry, his steady, keen interest in everything biological, his strict integrity of character; and the Board of Trustees will be very conscious of the absence of his sincere interest in the ongoing of the Laboratory, its policies, and its scientific significance.

Grave was born a Hoosier, on a farm in Monrovia, Indiana, and was very nearly 74 years of age at the time of his death at his home in Winter Park, Florida, last January 8th. He graduated from Earlham College in 1895, with Phi Beta Kappa rank; his alma mater honored him with her Doctor of Laws degree in 1928. His graduate studies were done at Johns Hopkins University, which conferred the Ph.D. in 1899. Meanwhile, he had spent summers at the Fisheries Bureau in Woods Hole, and at the Johns Hopkins Laboratory in Jamaica. After two further years of study as Bruce Fellow, he was appointed to the Hopkins teaching staff, a relation he continued for 18 years, for 13 of which he held the rank of Associate Professor. In 1919 he was appointed to the headship of the Zoological Department at Washington University, St. Louis, where the new Rebstock Laboratory had just been built. Here he gathered about him a staff of men of outstanding competency, and put the department on a basis widely recognized for scholarship and general efficiency.

Other responsibilities carried by Grave were: Director of the U. S. Fisheries Laboratory, Beaufort, N. C., 1902-1906; Shellfish Commissioner of Maryland, 1906-1912. In World War I he was ranked a captain in the Chemical Warfare Service. He was an active member of the AAAS; the American Society of Naturalists; a member of Sigma Xi; by turn he was Secretary-Treasurer, Vice President, and President of the American Society of Zoologists. As for the Marine Biological Laboratory, he was an outstandingly successful director of the Invertebrate Course from 1912-1917; a Trustee for 20 years, 1920-1940; thereafter, Trustee Emeritus. Few, if any, have taken the welfare of the Laboratory more seriously to heart than did he.

Grave's research interest embraced three quite different fields: pelecypod mollusca as to structure, physiology, and life histories; echinoderms, with special reference to embryology, and intraphyla relationships; while in later years he attacked the problem of metamorphosis in the ascidians, with special reference to the chemical factors retarding or accelerating it.

It is not too much to say that Caswell Grave was a wise man; and in the truest sense, in all that the appellations should imply, he was a "gentleman and a scholar." We are glad to pause and offer him such honor as we may, today.

August 9, 1944

## 2. MINUTES OF THE COMMITTEE OF REVIEW OF THE MARINE BIOLOGICAL LABORATORY

The Committee of Review provided for in the Deed of Trust Covering Funds for Endowment, Friendship Fund, Inc., and Central Hanover Bank and Trust Company of New York, met at the Marine Biological Laboratory, Woods Hole, Massachusetts, on August 9, 1944, at 9:00 A.M.

Mr. Lawrason Riggs, President of the Corporation, read the Call of Meeting, and commented on the history of the origin of the Deed of Trust, and on the duties of the Committee.

### Present:

Professor W. C. Allee—representing The University of Chicago  
Professor G. A. Baitsell—representing Yale University

Professor A. F. Blakeslee—representing The American Association for the Advancement of Science

Professor A. B. Dawson—representing Harvard University

Professor W. K. Gregory—representing The National Academy of Science

Professor R. W. Griggs—representing The National Research Council

Professor E. N. Harvey—representing Princeton University

Professor M. H. Jacobs—representing The University of Pennsylvania

Professor Franz Schrader—representing Columbia University

Dr. Blakeslee was elected Chairman of the Committee and (by invitation) Dr. Charles Packard, Director of the Laboratory, Secretary.

Dr. Packard presented the Decennial Review containing a brief statement of the activities of the Laboratory for the years 1934–1943, and called attention to the nine exhibits.

The Committee examined the exhibits, and after full discussion, unanimously VOTED That the Marine Biological Laboratory is performing valuable services in biological research.

It was the opinion of the Committee that it could perform a useful service to the Laboratory by making suggestions regarding its future development.

VOTED That the Committee understands and appreciates the high quality of the Board of Trustees of the Laboratory, but thinks it desirable that each class of Trustees should contain at least one biologist not closely associated with the work of the Laboratory.

Moved and seconded that a recommendation be formulated that some means be considered for effecting more frequent changes in the Board of Trustees.

The motion was lost.

VOTED That the Chairman appoint a sub-committee of three to report to the full Committee on the specific needs of the Laboratory.

The Chairman appointed Drs. Harvey, Dawson, and Packard.

VOTED That the Chairman appoint a sub-committee of three to draft a statement in support of the first motion, this to follow in general the form of the report of the 1934 Committee of Review.

The Chairman appointed Drs. Schrader, Jacobs, and Baitsell.

#### Afternoon Session.

VOTED To accept and adopt the following statement in support of the first motion.

The Marine Biological Laboratory is performing valuable services in biological research. Its record is especially commendable in view of the difficult conditions experienced during the past ten years. Despite the steady decrease in income from endowments, and the more recent handicaps involved in war conditions, the scientific activities of the Marine Biological Laboratory have been maintained at a high level.

With marked decrease in attendance due to wartime conditions, the standards of the courses of instruction have been maintained.

The Library, already recognized as one of the foremost in its field, has on a reduced budget been steadily improved.

Important research continues to be done. To compensate for a decrease in attendance there has been some utilization of the Laboratory facilities for war work.

As in the past, one of the important features of the Marine Biological Laboratory has been the close association of investigators working in different fields. Likewise, cooperation and association with the Woods Hole Oceanographic Institution, as well as with the local station of the U. S. Fish and Wild Life Service, has increased to a laudable extent.

VOTED To accept and adopt, as amended, the following statement of the sub-committee on Laboratory needs:

1. The committee notes that the income of the Marine Biological Laboratory has decreased while the needs have continually mounted. The budget has been balanced at the expense of upkeep and necessary improvements. Obviously the setting up of a sufficient reserve for future developments has been impossible. Additional income is urgently needed for the following specific purposes:
  - a. Replacement of apparatus, boats, and other equipment now becoming obsolete.
  - b. Repair and renovation of buildings.
  - c. Payment of subscriptions to foreign journals now held in Europe.
  - d. Probable adjustment of salaries to meet increased cost of living.
  - e. Additional pensions.
  - f. A naturalist to replace Mr. G. M. Gray, now retired.
  - g. A fireproof building to replace the present wooden Laboratory buildings.
2. The Committee recognizes that the acquisition of funds for the above purposes and for additional endowment constitutes the most important problem confronting the Trustees of the Laboratory. In view of the anticipated increase in research activity after the war, these needs appear to be immediate and imperative.

The Committee directed the Secretary to inform the Trustees of the Laboratory of the above matters. The condensed report will be forwarded to the Bank as Trustee of the Endowment Funds; the full minutes will be published in the 1944 Annual Report of the Director.

The Committee adjourned at 4:45 P.M.

CHARLES PACKARD,  
*Secretary*

August 9, 1944

### 3. TO THE COMMITTEE OF REVIEW

*Gentlemen:*

The first decennial review (1923-1933) included the period of rapid growth of the Laboratory. The Endowment Fund was set up; the chief building erected, more than doubling the space available for research; a special endowment for the

Library permitted a notable addition to its holdings of journals and books; a large amount of apparatus and other tools of research became available. As a result, the scientific activity of the Laboratory increased greatly. Toward the end of the period, the economic depression brought about a temporary slowing down of growth.

In the period now under review (1934-1943) growth was resumed. The Library overflowed the space allotted to it and spread into the new wing, a gift of the Rockefeller Foundation. The number of investigators increased, exceeding all previous records. The war has temporarily ended this growth. The Library now receives few foreign journals; the younger investigators are in active service or in war research; the classes, which have been maintained without interruption, are almost devoid of men. But the Laboratory has continued to offer all of its usual facilities to investigators and students. The current year (1944) shows a marked upward trend in attendance and scientific activity. The stability of the Laboratory during these periods of war and economic depression is noteworthy.

### Personnel

Many important changes in personnel have occurred in the past ten years. Dr. F. R. Lillie retired as President of the Corporation, and was elected President Emeritus. In his stead, Mr. Lawrason Riggs, the Treasurer since 1924, was chosen President; and the office of Vice President, created in 1942, was filled by Dr. E. N. Harvey.

The following changes have occurred in the Board of Trustees:

- (a) Died in Office: C. R. Stockard, D. H. Tennent.
- (b) Elected Trustees Emeritus (having reached the age of seventy years):

|                         |                        |
|-------------------------|------------------------|
| G. N. Calkins, d. 1943  | H. S. Jennings         |
| E. G. Conklin           | C. E. McClung          |
| B. M. Duggar            | S. O. Mast             |
| W. E. Garrey            | A. P. Mathews          |
| Caswell Grave, d. 1944  | W. J. V. Osterhout     |
| M. J. Greenman, d. 1938 | G. H. Parker           |
| R. G. Harrison          | W. M. Wheeler, d. 1937 |

- (c) Elected Trustees:

E. G. Ball, Assoc. Prof. Biol. Chem., Harvard Medical School  
 S. C. Brooks, Prof. of Zoology, University of California  
 D. E. S. Brown, Prof. of Physiology, N. Y. University Dental School  
 G. H. A. Clowes, Director of Research, Eli Lilly Laboratory  
 E. F. DuBois, Prof. of Physiology, Cornell Medical College  
 P. S. Galtsoff, Senior Biologist, U. S. Fish and Wild Life Service  
 Laurence Irving, Prof. of Biology, Swarthmore College  
 Columbus Iselin, Director, Woods Hole Oceanographic Institution  
 C. W. Metz, Prof. of Zoology, University of Pennsylvania  
 J. H. Northrup, Member, Rockefeller Institute

H. H. Plough, Prof. of Biology, Amherst College  
Franz Schrader, Prof. of Zoology, Columbia University  
E. W. Sinnott, Prof. of Botany, Yale University  
A. H. Sturtevant, Prof. of Genetics, Calif. Institute Technology  
W. R. Taylor, Prof. of Botany, University of Michigan  
B. H. Willier, Prof. of Zoology, Johns Hopkins University

Dr. M. H. Jacobs, appointed Director in 1926, resigned in 1937. Dr. Charles Packard was made Assistant Director in that year, and Director in 1939. Since 1942 he has been Resident Director.

Our investigators and students are drawn from institutions widely distributed throughout the country (cf. map, Exhibit 3). In addition to universities and colleges, 36 Medical Schools and Hospitals have sent representatives; 9 Research Institutes, a number of Federal and State services, and industrial laboratories are also represented. A complete list of all of these various institutions is found in Exhibits 4 and 5.

Statistics of attendance for the period under review are shown in Exhibit 3. The chart indicates the annual attendance since 1888 when the Laboratory was founded. The term "New Investigators" refers to those who work here for the first time; "Returning Investigators" are those who have previously spent one or more seasons at the Laboratory. The effect of the first world war and of the present war; of periods of economic depression; and of expansion in research facilities, can be seen.

An incomplete list of publications from this Laboratory is found in Exhibit 8. The scientific record of students attending the courses for the years 1918-1931 is also a part of this Exhibit since it indicates their continuing interest and success in biological research and teaching.

The Laboratory is in full operation and is open for your inspection.

Respectfully submitted,

LAWRASON RIGGS, *President*  
CHARLES PACKARD, *Director*

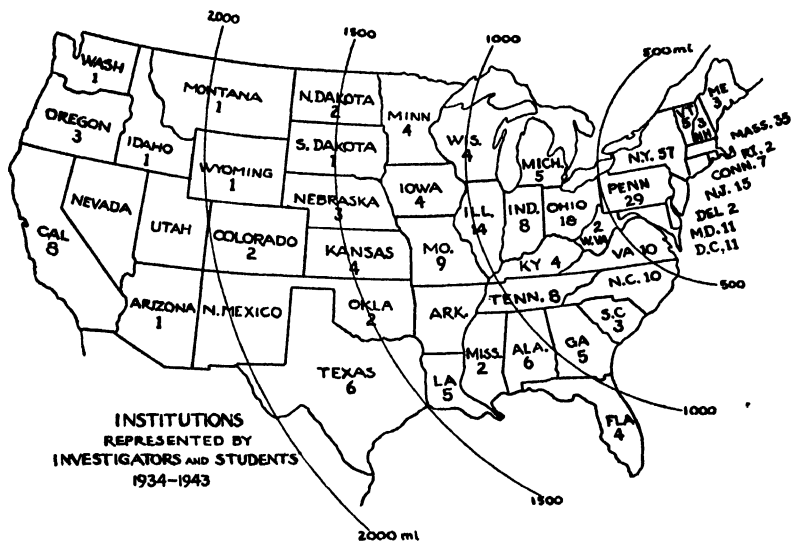
## EXHIBITS

*For the years 1934-1943 inclusive*

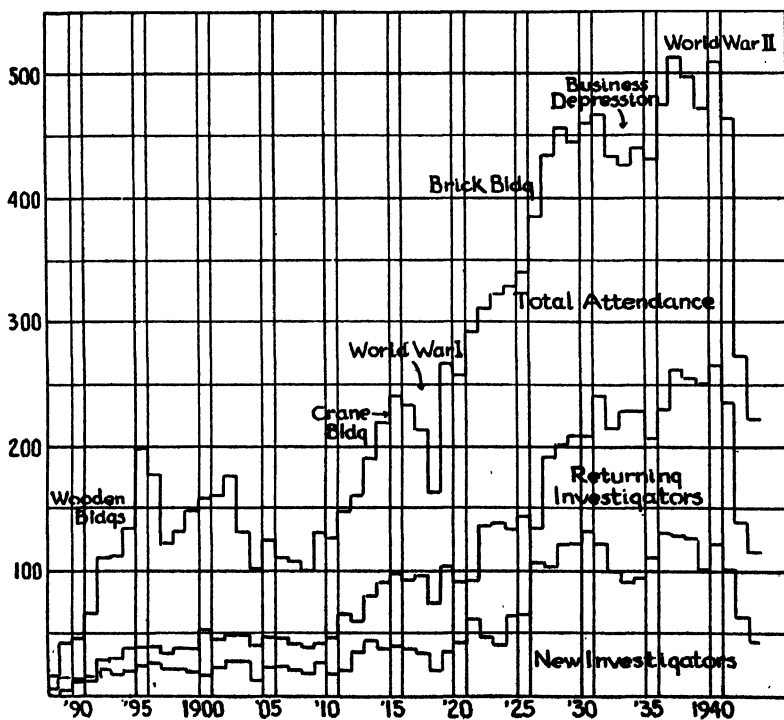
1. Annual Reports
2. Annual Announcements
- \*3. Statistics of Attendance
- \*4. Institutions represented by Investigators and Students
- \*5. Subscribing and Cooperating Institutions
- \*6. Additions to the Library. Check List of Journals
7. Catalog of Investigators
8. Partial List of Publications from the Laboratory
9. The Scientific Record of Students attending the Courses

\* These exhibits appear in this Report.

## MARINE BIOLOGICAL LABORATORY



Geographical distribution of institutions represented at the Marine Biological Laboratory 1934-1943



Attendance at the Marine Biological Laboratory 1888-1943

EXHIBIT 3  
A TABULAR VIEW OF ATTENDANCE 1934-1943

|  | 1934 | '35 | '36 | '37 | '38 | '39 | '40 | '41 | '42 | '43 |
|--|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Investigators—Total . . . . .              | 323  | 315 | 359 | 391 | 380 | 352 | 386 | 337 | 201 | 160 |
| Independent . . . . .                      | 222  | 208 | 226 | 256 | 246 | 213 | 253 | 197 | 132 | 89  |
| Beginning . . . . .                        | 49   | 56  | 76  | 74  | 53  | 60  | 62  | 59  | 16  | 19  |
| Research Ass'ts . . . . .                  | 52   | 51  | 57  | 61  | 81  | 79  | 71  | 50  | 25  | 17  |
| Library Readers . . . . .                  |      |     |     |     |     |     |     | 31  | 28  | 35  |
| Students—Total . . . . .                   | 131  | 130 | 138 | 133 | 132 | 133 | 128 | 131 | 74  | 68  |
| Botany . . . . .                           | 13   | 6   | 10  | 9   | 12  | 9   | 10  | 15  | 8   | —   |
| Embryology . . . . .                       | 30   | 33  | 34  | 35  | 34  | 36  | 34  | 37  | 24  | 13  |
| Physiology . . . . .                       | 23   | 20  | 22  | 16  | 22  | 21  | 22  | 24  | 6   | 8   |
| Protozoology . . . . .                     | 11   | 16  | 17  | 16  | 10  | 12  | 7   | —   | —   | —   |
| Zoology . . . . .                          | 54   | 55  | 55  | 57  | 54  | 55  | 55  | 55  | 36  | 47  |
| Total Attendance less double registrations | 439  | 429 | 473 | 511 | 496 | 471 | 507 | 461 | 273 | 222 |
| Institutions represented . . . . .         | 131  | 143 | 158 | 165 | 151 | 162 | 148 | 144 | 126 | 116 |
| By investigators . . . . .                 | 98   | 111 | 120 | 134 | 125 | 132 | 112 | 102 | 83  | 71  |
| By students . . . . .                      | 75   | 70  | 77  | 79  | 67  | 72  | 79  | 72  | 43  | 41  |
| Schools and Academies                      |      |     |     |     |     |     |     |     |     |     |
| By investigators . . . . .                 | 1    | —   | 2   | 3   | 4   | 2   | 1   | 5   | 2   | 1   |
| By students . . . . .                      | 5    | 3   | 3   | 2   | 1   | 2   | 2   | 2   | —   | 1   |
| Foreign Institutions                       |      |     |     |     |     |     |     |     |     |     |
| By investigators . . . . .                 | 4    | 7   | 9   | 16  | 14  | 8   | 2   | 3   | —   | 2   |
| By students . . . . .                      | 1    | 1   | 5   | —   | 3   | 1   | 1   | 1   | —   | —   |

## EXHIBIT 4

## INSTITUTIONS REPRESENTED BY INVESTIGATORS AND STUDENTS 1934-1943

## A. UNIVERSITIES AND COLLEGES

The figures after the name of the institution refer to the year the institution was first represented at the Laboratory

|                            |           | 1934 | '35 | '36 | '37 | '38 | '39 | '40 | '41 | '42 | '43 |
|----------------------------|-----------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Adelphi College            | N. Y. '38 |      |     |     |     | 1   |     |     |     |     |     |
| Agnes Scott College        | Ala. '15  |      | 1   | 1   |     | 1   | 1   |     |     | 1   |     |
| Akron, University of       | Ohio '15  | 1    |     |     |     |     |     |     |     |     |     |
| Alabama, University of     | Ala. '20  |      | 1   | 1   | 2   |     |     | 1   | 2   |     |     |
| Alabama University Medical | '37       |      |     |     | 1   | 1   |     |     |     |     |     |
| Alabama Polytech. Inst.    | Ala. '38  |      |     |     |     | 1   |     |     |     |     |     |
| Albany Medical Coll.       | N. Y. '31 |      |     |     |     |     | 1   | 1   |     |     |     |
| Albion College             | Mich. '92 |      | 2   | 1   | 1   |     | 1   |     |     |     |     |
| American Internat. Coll.   | Mass. '42 |      |     |     |     |     |     |     |     | 1   |     |
| American University        | D. C. '31 | 3    | 2   | 2   | 1   | 1   |     |     | 1   |     |     |
| Amherst College            | Mass. '13 | 2    | 5   | 11  | 10  | 6   | 8   | 6   | 6   | 7   | 1   |
| Antioch College            | Ohio '23  |      |     |     | 1   |     |     |     |     |     |     |
| Arizona, University of     | Ariz. '25 |      |     |     |     |     |     | 1   |     |     |     |
| Assumption College         | Mass. '42 |      |     |     |     |     |     |     |     | 1   |     |
| Atlanta University         | Ga. '34   | 1    | 1   | 3   | 1   |     | 1   |     |     | 1   |     |
| Baldwin-Wallace Coll.      | Ohio '35  |      | 1   |     |     |     |     |     |     |     |     |
| Bard College               | N. Y. '35 |      | 1   | 1   | 2   | 1   |     |     |     |     |     |
| Barnard College            | N. Y. '96 | 6    | 3   | 4   | 4   | 3   | 1   | 3   | 2   |     | 1   |
| Baylor Univ. Medical       | Tex. '42  |      |     |     |     |     |     |     |     | 1   |     |
| Beloit College             | Wis. '96  |      |     |     |     |     |     | 1   |     |     |     |
| Bennington College         | Vt. '35   |      | 2   |     |     |     |     | 1   |     | 1   | 1   |
| Berea College              | Ky. '28   |      | 1   |     |     |     |     |     |     |     |     |
| Birmingham-South. Univ.    | Ala. '26  |      |     |     |     |     | 1   |     |     |     |     |
| Boston College             | Mass. '38 |      |     |     |     | 1   |     |     |     |     |     |
| Boston University          | Mass. '17 |      |     |     |     |     | 1   |     |     |     |     |
| Boston University Medical  | Mass. '37 |      |     |     | 1   |     |     |     |     |     |     |
| Boston Teachers Coll.      | Mass. '37 |      |     |     | 1   |     |     |     |     |     |     |
| Bowdoin College            | Me. '93   | 2    | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   |     |
| Bradley Poly. Inst.        | Ill. '96  | 1    |     |     |     |     |     |     |     |     |     |
| Bridgewater State T. C.    | Mass. '38 |      |     |     |     | 2   |     |     |     |     |     |
| Brooklyn College           | N. Y. '32 | 7    | 5   | 9   | 7   | 2   | 4   | 7   | 3   | 1   | 1   |
| Brown University           | R. I. '90 | 1    |     | 1   | 3   | 3   | 7   | 7   | 7   | 1   |     |

## EXHIBIT 4—Continued

|  |                        | 1934    | '35     | '36    | '37     | '38    | '39     | '40     | '41    | '42    | '43    |
|--|------------------------|---------|---------|--------|---------|--------|---------|---------|--------|--------|--------|
| Bryn Mawr College<br>Buffalo, University of        | Penn. '88<br>N. Y. '95 | 1       | 1       |        | 1       |        | 5<br>2  | 2       | 4      |        |        |
| Buffalo University Medical<br>Butler University    | N. Y. '39<br>Ind. '16  |         | 2       | 1      |         |        | 1       |         |        |        |        |
| California Inst. Tech.<br>California, Univ. of     | Cal. '29<br>Cal. '00   | 2       | 3<br>1  | 2<br>3 | 6       | 3<br>2 | 3<br>3  | 2<br>5  | 4<br>4 | 2<br>5 | 2<br>1 |
| Canisius College<br>Carnegie Inst. Tech.           | N. Y. '37<br>Penn. '09 | 1       |         |        | 1<br>1  | 1      | 2       | 4       | 2      | 4      |        |
| Catholic Univ. of Amer.<br>Central State T. C.     | D. C. '42<br>Okla. '36 |         |         | 1      |         |        |         |         |        | 2      | 2      |
| Centre College<br>Charleston, Coll. of             | Ky. '37<br>S. C. '06   |         |         | 1      | 1       |        | 1       | 1       |        |        | 1      |
| Chestnut Hill College<br>Chicago, University of    | Penn. '41<br>Ill. '92  | 10      | 7       | 6      | 7       | 9      | 5       | 13      | 2<br>8 | 2<br>2 | 3      |
| Chicago University Medical<br>Cincinnati, Univ. of | Ill. '40<br>Ohio '92   | 7       | 9       | 7      | 8       | 7      | 5       | 1<br>3  | 1<br>7 |        | 1      |
| Cincinnati Univ. Medical<br>Clark University       | Ohio '35<br>Mass. '88  | 1       | 3<br>2  | 2<br>1 | 3<br>1  | 3<br>1 | 5<br>2  | 3       | 1      |        |        |
| Coe College<br>Colby College                       | Iowa '20<br>Me. '99    |         |         | 1      |         |        |         |         |        | 1      |        |
| Colgate University<br>College of Scholastica       | N. Y. '98<br>Minn. '39 |         |         |        |         | 1      | 1       |         |        |        |        |
| College City of N. Y.<br>Colorado, Univ. of        | N. Y. '94<br>Col. '14  | 7<br>2  | 2<br>1  | 6      | 8       | 5      | 3       | 5       | 5      |        |        |
| Colorado Univ. Medical<br>Columbia University      | Col. '25<br>N. Y. '91  | 28      | 25      | 22     | 20      | 21     | 18      | 1<br>18 | 11     | 8      | 5      |
| Columbia University Medical<br>Connecticut College | N. Y. '94<br>Conn. '20 | 5       | 5       | 5<br>2 | 7<br>2  | 8<br>2 | 5<br>3  | 3<br>3  | 2<br>2 | 5<br>2 | 4<br>1 |
| Converse College<br>Cornell University             | S. C. '38<br>N. Y. '91 | 1       |         | 2      | 2       | 1<br>3 | 3       | 3       | 5      | 4      | 1      |
| Cornell University Medical<br>Dartmouth College    | N. Y. '09<br>N. H. '96 | 12<br>4 | 12<br>3 | 9<br>4 | 11<br>4 | 8<br>4 | 10<br>4 | 6<br>4  | 5<br>3 | 1<br>2 | 1<br>1 |
| Davis and Elkins Coll.<br>Delaware, Univ. of       | W. Va. '41<br>Del. '98 |         |         |        |         |        |         | 1       | 1      |        |        |
| Delta State T. C.<br>DePaul University             | La. '36<br>Ill. '41    |         |         | 1      |         |        |         |         | 1      |        |        |

[illegible]

## EXHIBIT 4—Continued

|  |                        | 1934   | '35    | '36    | '37     | '38     | '39     | '40     | '41     | '42    | '43    |
|--|------------------------|--------|--------|--------|---------|---------|---------|---------|---------|--------|--------|
| Hood College<br>Howard University                    | Md. '15<br>D. C. '09   | 1<br>1 | 1      | 1      | 2       |         | 1       |         |         |        |        |
| Hunter College<br>Illinois, Univ. of                 | N. Y. '14<br>Ill. '90  | 7<br>5 | 3<br>6 | 4<br>2 | 2<br>4  | 1<br>2  | 4<br>1  | 1<br>5  |         | 3<br>3 |        |
| Indiana University<br>Iowa, State Univ. of           | Ind. '89<br>Iowa '94   |        |        | 1<br>6 |         |         |         | 2<br>5  | 2<br>6  |        | 1      |
| Iowa, State College of<br>Johns Hopkins Univ.        | Iowa '19<br>Md. '89    | 2<br>9 | 1<br>3 | 3<br>8 | 3<br>11 | 3<br>17 | 5<br>12 | 1<br>10 | 1<br>11 |        | 2      |
| Johns Hopkins Univ. Medical<br>Kansas, University of | Md.<br>Kan. '90        | 2<br>1 | 2      | 4<br>2 | 2<br>1  | 4       | 5       | 1<br>1  |         |        |        |
| Kansas State College<br>Kansas State T. C.           | Kan. '26<br>Kan. '34   |        | 1      |        |         |         |         |         |         | 1      |        |
| Kent State University<br>Kenyon College              | Ohio '30<br>Ohio '96   |        |        |        | 1       | 1       |         | 1       |         |        |        |
| Lander College<br>LaVerne College                    | S. C. '38<br>Cal. '34  | 1      |        |        |         | 1       |         |         |         |        |        |
| Leland Stanford Univ.<br>Lincoln University          | Cal. '91<br>Penn. '01  |        | 1      | 1      |         | 1       | 3       | 7       | 1       | 1      |        |
| Long Island Univ.<br>Long Island Univ. Medical       | N. Y. '29<br>N. Y. '19 | 3      | 3      | 3      | 2       | 1       | 1       | 1       |         | 1<br>1 |        |
| Loyola Univ. Medical<br>Maine, University of         | La. '31<br>Me. '95     | 1<br>1 |        | 1<br>1 | 1       |         |         |         | 1       |        |        |
| Marquette University<br>Maryland, Univ. of           | Wis. '36<br>Md. '41    |        |        | 1      |         |         |         |         | 1       |        |        |
| Maryland Univ. Medical<br>Mass. State College        | Md. '96<br>Mass. '89   |        |        | 8      | 11<br>1 | 13<br>1 | 11<br>1 | 4<br>1  | 3<br>2  | 5<br>2 | 1<br>1 |
| Mass. Inst. Technology<br>Miami University           | Mass. '88<br>Ohio '91  | 1      |        |        | 1       | 3       | 1<br>3  | 1<br>3  | 2<br>3  | 2<br>2 | 1      |
| Michigan, Univ. of<br>Michigan Agric. Coll.          | Mich. '88<br>Mich. '10 | 4      | 3      | 2      | 2       | 4<br>1  | 3       | 6       | 9       | 2      | 2      |
| Middlebury College<br>Middlesex College              | Vt. '95<br>Mass. '37   |        | 1      |        | 1       | 1       | 1       |         |         |        |        |
| Minnesota, Univ. of<br>Mississippi, Univ. of         | Minn. '98<br>Miss. '99 |        | 1      | 2      | 7       | 3       | 2       | 3<br>1  | 2       | 1      |        |
| Missouri, Univ. of<br>Missouri State T. C.           | Mo. '95<br>Mo. '43     | 3      | 7      | 10     | 1       | 7       | 1       | 5       | 4       | 1      | 1      |

EXHIBIT 4—*Continued*

|  |                        | 1934   | '35    | '36     | '37     | '38     | '39     | '40     | '41     | '42    | '43    |
|--|------------------------|--------|--------|---------|---------|---------|---------|---------|---------|--------|--------|
| Moberly Junior Coll.<br>Morehouse College                | Wis. '40<br>Ga. '27    |        |        |         |         |         | 1       | 1       |         |        |        |
| Morristown College<br>Mt. Holyoke College                | Tenn. '37<br>Mass. '88 | 3      | 3      | 6       | 1<br>7  | 8       | 7       | 4       | 8       | 8      | 3      |
| Mt. Mercy College<br>Mt. St. Louis Coll.                 | Penn. '39<br>'34       | 1      |        |         |         |         | 1       |         |         |        |        |
| Mundelein University<br>National Park Coll.              | Ill. '39<br>Md. '42    |        |        |         |         |         | 1       |         | 1       | 1      |        |
| Nebraska, Univ. of Medic.<br>New Hampshire State U.      | Neb. '34<br>N. H. '00  | 1      |        |         |         | 1       |         |         |         |        |        |
| New Jersey College for Women                             | N. J. '28              | 4      | 3      | 2       | 1       | 3       | 1       |         | 1       |        | 1      |
| New Jersey State T. C.<br>New Rochelle, Coll. of         | N. J. '31<br>N. Y. '34 | 2<br>1 | 4      | 6       | 5       | 3<br>1  | 4<br>1  | 3       | 1       |        | 1      |
| New York University<br>New York University Medical       | N. Y. '96<br>N. Y. '25 | 4<br>6 | 3<br>2 | 10<br>6 | 7<br>10 | 7<br>7  | 2<br>3  | 5<br>9  | 7<br>5  | 2<br>7 | 2<br>6 |
| New York University Wash. Sq.<br>Newark, University of   | N. Y. '24<br>N. J. '41 | 10     | 10     | 10      | 10      | 11      | 10      | 12      | 8<br>1  | 9      | 5      |
| Newark State T. C.<br>North Carolina, Univ. of           | N. J. '41<br>N. C. '99 |        | 1      | 1       | 1       | 2       | 3       | 1       | 1<br>3  |        | 3      |
| North Carolina Coll. for Negroes                         | N. C. '40              |        |        |         |         |         |         | 1       |         |        |        |
| N. C. State College<br>N. C. Womens College              | N. C. '31<br>N. C. '22 |        | 1      | 2<br>1  | 1       |         |         |         |         |        |        |
| N. Dakota State Univ.<br>N. Dakota Agric. Coll.          | N. D. '93<br>N. D. '23 | 2      | 1<br>1 | 1       |         |         |         | 1       |         |        |        |
| N. Texas Agric. Coll.<br>Northwestern Univ.              | Tex. '38<br>Ill. '93   | 3      | 4      | 3       | 6       | 1<br>1  | 5       | 9       | 2       |        |        |
| Notre Dame Univ.<br>Oberlin College                      | Ind. '21<br>Ohio '90   | 5      | 7      | 1<br>8  | 1<br>7  | 1<br>5  | 1<br>4  | 6       | 6       | 4      | 5      |
| Ohio State Univ.<br>Ohio University                      | Ohio '90<br>Ohio '14   |        |        | 1       | 4<br>2  | 10      | 3       | 7       | 8       | 5      | 2      |
| Ohio Wesleyan Univ.<br>Oklahoma, Univ. of                | Ohio '91<br>Okla. '09  |        |        |         | 1       | 1       | 2<br>1  | 2<br>1  | 2       |        | 1      |
| Oklahoma City, Univ. of<br>Pennsylvania, Univ. of        | Okla. '37<br>Penn. '91 | 37     | 38     | 35      | 1<br>31 | 1<br>28 | 1<br>26 | 1<br>32 | 1<br>27 | 17     | 17     |
| Pennsylvania, Univ. of 'Medical<br>Penn. Coll. for Women | Penn. '01              | 6<br>3 | 3<br>3 | 5<br>1  | 6<br>1  | 5<br>2  | 8<br>2  | 8<br>2  | 6<br>2  | 6<br>2 | 3<br>1 |

## EXHIBIT 4—Continued

|  |                        | 1934   | '35     | '36     | '37    | '38    | '39    | '40    | '41    | '42    | '43 |
|--|------------------------|--------|---------|---------|--------|--------|--------|--------|--------|--------|-----|
| Penn. State Coll.<br>Pittsburgh, Univ. of                    | Penn. '07<br>Penn. '21 | 3      | 5       | 7       | 7      | 4      | 1<br>5 | 7      | 6      | 2      |     |
| Placer Junior College<br>Pomona College                      | Cal. '40<br>Cal. '24   |        |         | 1       |        |        |        | 1      |        |        |     |
| Princeton University<br>Providence College                   | N. J. '90<br>R. I. '35 | 12     | 7<br>1  | 12<br>2 | 13     | 12     | 13     | 9      | 9      | 3      | 5   |
| Purdue University<br>Queens College                          | Ind. '28<br>N. Y. '28  |        | 3       | 5       | 4      | 5<br>1 | 1<br>1 | 3<br>4 | 3<br>3 |        |     |
| Radcliffe College<br>Randolph-Macon Coll.                    | Mass. '95<br>Va. '89   | 4      | 3       | 3       | 6      | 2      | 1      | 2      | 5<br>1 | 2      | 1   |
| Reed College<br>Rensselaer Poly. Inst.                       | Ore. '39<br>N. Y. '36  |        |         | 1       | 1      |        | 1      |        | 1      |        |     |
| Rice Institute<br>Richmond, College of                       | Tex. '16<br>Va. '13    |        |         |         | 1<br>1 | 1      |        |        | 1      | 1      | 1   |
| Rochester, Univ. of<br>Rochester, Univ. of Medical           | N. Y. '92<br>N. Y. '35 | 8      | 10<br>5 | 4       | 7<br>2 | 5<br>2 | 5<br>1 | 6      | 6      | 2      | 4   |
| Russell Sage College<br>Rutgers University                   | N. Y. '20<br>N. J. '14 |        |         | 2       | 1<br>1 |        | 2<br>2 | 2<br>1 | 1<br>2 | 5<br>2 | 4   |
| St. Francis Xavier Coll.<br>St. Johns College                | Ill. '14<br>Md. '34    | 1<br>2 | 2       | 2<br>1  | 2      | 2      | 1      |        |        |        |     |
| St. Louis University<br>St. Louis University, Maryville      | Mo. '03<br>Mo. '37     | 1      | 1       | 2       | 3<br>1 |        |        |        |        |        |     |
| St. Thomas College<br>St. Vincent College                    | Minn. '35<br>Penn. '24 |        | 1       |         |        |        |        |        | 1      |        |     |
| Sarah Lawrence College<br>Seton Hall College                 | N. Y. '32<br>N. J. '35 |        | 4<br>1  | 5<br>2  | 1      |        |        |        | 2      | 1      |     |
| Seton Hill College<br>Simmons College                        | Penn. '29<br>Mass. '07 | 1      | 1       | 1       | 1<br>1 | 1<br>1 |        | 1<br>1 | 3      | 1      | 4   |
| Skidmore College<br>Smith College                            | N. Y. '22<br>Mass. '92 | 1<br>1 | 2<br>7  | 2<br>5  | 2<br>2 | 2      | 2      | 2      | 1<br>5 | 1<br>4 | 3   |
| J. C. Smith University<br>Southern California, University of | N. C. '34<br>Cal. '96  | 1      | 1       | 1       | 1      | 2      | 2      |        |        |        |     |
| Southern Oregon State Normal                                 | Ore. '38               |        |         |         |        | 1      |        |        |        |        |     |
| Southwestern Univ.<br>Spring Hill College                    | Tenn. '22<br>Ala. '38  |        | 1       |         |        | 1      |        | 1      |        |        |     |
| Springfield College<br>Stephens College                      | Mass. '40<br>Mo. '36   |        |         | 1       |        |        |        | 2      | 1      |        |     |

EXHIBIT 4—*Continued*

|                                  |           | 1934 | '35 | '36 | '37 | '38 | '39 | '40 | '41 | '42 | '43 |
|----------------------------------|-----------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Swarthmore College               | Penn. '88 | 6    | 4   | 7   | 6   | 11  | 9   | 7   | 4   |     | 1   |
| Sweet Briar College              | Va. '09   |      | 2   |     |     |     |     |     |     | 1   |     |
| Syracuse University              | N. Y. '94 |      | 1   | 1   | 2   |     |     | 1   | 1   | 1   |     |
| Syracuse University Medical      | N. Y. '99 | 2    | 2   | 1   | 1   | 1   | 2   | 4   | 2   | 1   |     |
| Temple University                | Penn. '97 | 2    | 2   | 1   | 4   | 3   | 2   | 3   | 4   | 2   | 3   |
| Tennessee, Univ. of              | Tenn. '95 | 3    |     |     |     |     |     |     |     |     |     |
| Tennessee, Univ. of Medical      | Tenn. '35 |      | 2   | 2   | 2   |     |     |     |     |     |     |
| Texas, University of             | Tex. '95  |      |     |     |     | 1   |     | 1   | 1   |     |     |
| Texas, University of Medical     | Tex. '36  |      |     | 1   | 1   | 1   |     |     |     |     |     |
| Texas Christian Univ.            | Tex. '21  |      |     |     |     |     |     |     |     | 1   |     |
| Toledo, Univ. of                 | Ohio '17  |      |     |     |     | 2   | 2   |     | 1   |     |     |
| Tougaloo College                 | Miss. '39 |      |     |     |     |     | 1   |     |     |     |     |
| Trinity College                  | Conn. '00 | 1    | 2   | 2   | 2   | 2   | 2   | 1   | 1   | 1   |     |
| Tufts College                    | Mass. '92 |      |     | 1   |     |     | 2   | 1   | 2   |     | 2   |
| Tulane University                | La. '16   |      |     |     |     |     |     |     | 1   |     |     |
| Tulane Newcomb Coll.             | La. '13   | 1    |     |     |     |     |     |     | 1   |     |     |
| Union College                    | N. Y. '15 | 2    | 1   | 2   | 1   | 3   | 2   | 4   | 3   | 2   | 1   |
| Union College                    | Ky. '39   |      |     |     |     |     | 1   |     |     |     |     |
| Ursinus College                  | Penn. '95 |      | 1   |     |     |     |     |     |     |     |     |
| Vanderbilt University            | Tenn. '91 |      | 1   |     |     |     |     | 1   |     |     |     |
| Vanderbilt University Medical    | Tenn. '34 | 4    | 3   | 3   | 3   | 4   | 3   | 2   | 1   | 2   | 1   |
| Vassar College                   | N. Y. '88 | 4    | 2   | 3   | 2   | 6   | 3   | 5   | 7   | 6   | 6   |
| Vermont, University of           | Vt. '15   |      | 1   |     |     |     |     | 1   |     |     |     |
| Vermont, University of Medical   | Vt. '38   |      |     |     |     | 2   | 1   | 1   | 1   | 1   | 1   |
| Vermont State Normal             | Vt. '40   |      |     |     |     |     |     | 1   | 1   | 1   | 1   |
| Villanova College                | Penn. '39 |      |     |     |     |     | 1   | 6   | 5   | 1   |     |
| Virginia, Univ. of               | Va. '91   |      | 2   | 1   | 1   | 2   | 2   |     |     |     |     |
| Virginia, Univ. of Medical       | Va.       | 2    | 2   | 2   | 4   | 2   | 2   | 2   | 1   |     |     |
| Virginia State T. C.             | Va. '34   | 1    |     |     |     |     |     |     |     |     |     |
| Virginia Intermont Coll.         | Va. '41   |      |     |     |     |     |     |     | 1   |     |     |
| Wabash College                   | Ind. '07  | 1    | 2   | 3   | 1   | 1   |     | 2   | 2   |     |     |
| Washburn College                 | Kan. 40'  |      |     |     |     |     |     | 1   |     |     |     |
| Washington University            | Mo. '00   | 1    | 2   | 8   | 12  | 9   | 10  | 8   | 5   | 5   | 9   |
| Washington University Medical    | Mo.       |      | 2   |     | 6   | 3   | 2   | 3   | 1   |     |     |
| Washington, Univ. of             | Wash. '15 |      |     |     |     | 1   |     |     |     |     |     |
| Washington and Jefferson College | Penn. '11 | 1    | 1   | 1   | 1   | 2   | 1   | 2   | 1   | 2   | 1   |

## EXHIBIT 4—Continued

|   |                         | 1934   | '35    | '36    | '37    | '38     | '39    | '40     | '41     | '42    | '43    |
|---|-------------------------|--------|--------|--------|--------|---------|--------|---------|---------|--------|--------|
| Washington and Lee<br>Wayne University        | Va. '17<br>Mich. '34    | 1      | 1      | 1      |        |         |        |         |         |        |        |
| Wellesley College<br>Wesleyan University      | Mass. '88<br>Conn. '89  | 3<br>6 | 5<br>5 | 3<br>3 | 3<br>5 | 4<br>6  | 2<br>4 | 2<br>7  | 2<br>10 | 5<br>3 | 1<br>1 |
| West Virginia Univ.<br>Westbrook Junior Coll. | W. Va. '01<br>Conn. '40 | 1      |        |        |        |         | 1      | 1       |         |        |        |
| Western College<br>Western Reserve Univ.      | Ohio '98<br>Ohio '95    | 3      | 1      |        |        | 1<br>4  | 1      |         | 1       |        |        |
| Wheaton College<br>Whitman College            | Mass. '18<br>Ore. '43   | 1      | 2      | 1      | 1      | 4       | 3      | 4       | 2       | 2      | 3<br>2 |
| William and Mary<br>Williams College          | Va. '22<br>Mass. '92    | 1      | 4      | 1<br>2 | 1<br>1 | 1<br>5  | 1<br>4 | 1<br>3  | 1<br>5  | 1      |        |
| Wilson College<br>Wisconsin, Univ. of         | Penn. '24<br>Wis. '98   | 1<br>4 | 1<br>4 | 2<br>2 | 2<br>5 | 2       | 1<br>1 | 1<br>2  | 1<br>2  | 1      | 1<br>2 |
| Womens Medical College<br>Wooster College     | Penn. '92<br>Ohio '13   | 1      |        | 3<br>1 | 3<br>1 |         | 2      | 3       |         |        |        |
| Wright Junior College<br>Wyoming, Univ. of    | Ill. '41<br>Wyo. '29    |        |        |        |        |         |        |         | 1<br>1  |        |        |
| Yale University<br>Yale University Medical    | Conn. '91<br>Conn. '38  | 14     | 8      | 6      | 16     | 10<br>1 | 9      | 11<br>4 | 15<br>2 | 5<br>1 | 1      |

## B. HIGH SCHOOLS, ACADEMIES, ETC.

|   |                | 1934   | '35 | '36    | '37 | '38 | '39    | '40 | '41 | '42 | '43 |
|---|----------------|--------|-----|--------|-----|-----|--------|-----|-----|-----|-----|
| Abraham Lincoln H. S.<br>Agnes Irwin School | N. Y.<br>N. Y. |        |     | 1<br>1 |     |     |        |     |     |     |     |
| Annapolis H. S.<br>Berkshire School         | Md.<br>Mass.   | 1      |     |        |     |     |        |     | 1   |     |     |
| Birmingham H. S.<br>Boston H. S.            | Ala.<br>Mass.  | 1<br>1 |     |        |     |     |        |     | 1   |     |     |
| Bronxville H. S.<br>Caldwell H. S.          | N. Y.<br>N. J. |        | 1   | 1      |     |     |        |     |     |     |     |
| Central H. S.<br>Chicago H. S.              | D. C.<br>Ill.  |        |     |        | 1   |     | 1      |     |     |     |     |
| Choate School<br>Dana Hall                  | Conn.<br>Mass. |        |     | 1      |     |     |        | 1   | 1   |     |     |
| Darrow School<br>Deerfield Academy          | N. Y.<br>Mass. |        |     |        |     |     | 1<br>1 |     |     |     |     |

EXHIBIT 4—*Continued*

|   |                | 1934   | '35    | '36 | '37 | '38 | '39 | '40    | '41    | '42 | '43 |
|---|----------------|--------|--------|-----|-----|-----|-----|--------|--------|-----|-----|
| Eastern District H. S.<br>Emma Willard School     | N. Y.<br>N. Y. |        |        |     | 1   |     |     |        |        |     | 1   |
| Exeter Academy<br>Galesburg H. S.                 | N. H.<br>Ill.  | 1      |        |     |     |     |     | 1      | 1      | 1   | 1   |
| Grand Falls H. S.<br>Groton School                | Can.<br>Conn.  |        |        | 1   | 1   |     |     |        |        | 1   |     |
| Hallahan H. S.<br>Hawthorne H. S.                 | Penn.<br>N. J. |        |        | 1   |     |     |     | 1      |        |     |     |
| Hyde School<br>Hyde Park School                   | Mass.<br>Ill.  |        |        | 1   | 1   |     |     | 1      | 1      |     |     |
| Jenkintown H. S.<br>Knox School                   | Penn.<br>N. Y. |        | 1      | 1   |     |     |     |        |        |     |     |
| Lawrenceville School<br>Los Angeles H. S.         | Mass.<br>Cal.  |        |        |     |     |     |     |        | 1<br>1 |     |     |
| Milton Academy<br>Nativity H. S.                  | Mass.<br>Mass. | 1      |        | 1   |     | 4   | 4   | 5<br>1 | 4      |     |     |
| Negaunee H. S.<br>Oradell H. S.                   | Mich<br>N. J.  | 1      |        |     |     | 1   |     |        |        |     |     |
| Pennsgrove School<br>Potomac School               | Penn.<br>D. C. |        |        |     |     |     |     |        | 1<br>1 |     |     |
| St. Andrews School<br>St. Catherine School        | Del.<br>Va.    |        |        |     |     |     | 1   | 1      |        |     |     |
| St. Joseph's Seminary<br>St. Mary of the Lake Sem | N. Y.<br>Ill.  |        |        |     |     |     |     |        | 1      | 1   |     |
| Scott, H. S.<br>Society of the Divine Word        | Ohio<br>Mass.  | 1      |        |     |     |     |     |        |        | 1   |     |
| Straubenmiller H. S.<br>Theo. Roosevelt H. S.     | N. Y.<br>N. Y. |        |        | 1   | 1   |     |     |        |        |     |     |
| Union H. S.<br>Union City H. S.                   | N. J.<br>Tenn. | 1<br>1 |        |     |     |     |     |        |        |     |     |
| University H. S.<br>Vineland Training School      | Minn.<br>N. J. |        | 1<br>1 | 1   |     |     |     |        |        |     |     |
| Walton H. S.<br>Washington H. S.                  | N. J.<br>D. C. |        |        |     | 1   |     | 1   |        |        |     |     |
| Weequahic H. S.<br>Westtown Friends School        | N. J.<br>Penn. |        |        | 1   | 1   | 1   |     | 1      |        | 1   |     |

EXHIBIT 4—*Continued*

## C. INSTITUTES, FOUNDATIONS, ETC.

|  |                        | 1934 | '35 | '36 | '37 | '38     | '39 | '40     | '41    | '42    | '43    |
|--|------------------------|------|-----|-----|-----|---------|-----|---------|--------|--------|--------|
| American Mus. Nat. Hist.<br>Arlington Chemical Co                          | N. Y. '09<br>'31       | 1    | 1   |     |     |         |     |         |        |        |        |
| Barnard Skin and Cancer Hospital   | Mo.                    |      |     |     |     |         |     |         | 1      |        |        |
| Beth Israel Hospital<br>Biol. Institute, Phil.                             | N. Y. '42<br>Penn. '39 |      |     |     |     |         | 1   |         |        |        |        |
| Carnegie Institute<br>Cold Spring<br>Washington                            | N. Y. '14<br>D. C. '15 | 2    | 1   |     |     | 4       | 2   | 1       | 1      |        |        |
| Frick Education Comm.<br>Guggenheim Foundation<br>Guggenheim Dental Clinic | '42<br>'40<br>'43      |      |     |     |     |         |     | 1       |        | 1      | 1      |
| Journ. Industrial and Engineering<br>Chemistry                             | '28                    | 1    | 1   | 1   | 1   | 1       | 1   | 1       | 1      |        |        |
| Internat. Cancer Research Founda-<br>tion                                  | '37                    |      |     |     | 1   |         |     |         |        |        |        |
| Eli Lilly Company<br>Marine Studios, Inc.                                  | Ind. '19<br>Fla. '42   | 5    | 4   | 4   | 4   | 5       | 5   | 4       | 5      | 5      | 2      |
| Memorial Hospital<br>Mt. Sinai Hospital                                    | N. Y. '26<br>N. Y. '40 | 2    | 1   | 1   | 2   | 3       | 3   | 2<br>2  | 1<br>1 | 1<br>1 | 1<br>1 |
| Nat. Cancer Institute<br>Nat. Research Council                             | Md. '39<br>D. C.       |      |     |     | 1   |         | 2   |         |        | 1      |        |
| N. Y. State Agricult. Station<br>N. Y. State Dept. Health                  | N. Y. '18<br>N. Y. '19 | 1    | 2   | 1   | 1   | 1       | 1   | 2       | 2      | 1      |        |
| Overly Biochemical Research Found.   | N. Y. '43              |      |     |     |     |         |     |         |        |        | 1      |
| Phila. Acad. Nat. Sci.<br>Rockefeller Institute                            | Penn. '89<br>N. Y. '11 | 10   | 12  | 11  | 11  | 1<br>13 | 8   | 1<br>14 |        | 9      | 5      |
| Rockefeller Foundation Fellowship  |                        | 3    |     | 1   |     | 2       | 1   |         |        |        |        |
| Russell Sage Institute of Pathology  | N. Y. '34              | 1    |     |     |     |         | 1   | 1       |        |        |        |
| St. Luke's Hospital<br>U. S. Dept. Agriculture                             | N. Y. '40<br>D. C. '99 |      |     |     |     | 1       |     | 1       |        |        |        |
| U. S. Dept. Public Health<br>U. S. Fish and Wild Life Service              | D. C. '20<br>D. C. '42 |      |     |     |     | 1       |     |         |        | 2      | 2      |
| Wistar Institute<br>Woods Hole Oceanographic Inst.                         | Penn. '08<br>Mass. '43 | 1    | 1   | 1   | 1   |         | 1   |         |        |        |        |

## EXHIBIT 4—Continued

## D. INSTITUTIONS OUTSIDE THE UNITED STATES

|                   |  | 1934 | '35    | '36         | '37    | '38    | '39    | '40    | '41 | '42 | '43 |
|-------------------|--|------|--------|-------------|--------|--------|--------|--------|-----|-----|-----|
| Austria           | Univ. of Innsbruck<br>Univ. of Vienna                                      | 1    |        |             |        | 2      |        |        |     |     |     |
| Belgium           | Univ. of Ghent<br>Univ. of Liege<br>Belgian-American Educ. Founda-<br>tion |      |        |             |        | 1<br>1 | 3      |        |     |     |     |
| British<br>Isles  | Queens Coll. Belfast<br>Cambridge University                               | 1    |        | 1           | 1      | 1      | 1      |        |     |     |     |
|                   | Trinity Coll. Dublin<br>Univ. of Edinburgh                                 |      |        |             | 1      | 1      |        |        |     |     |     |
|                   | Univ. of Leeds<br>Univ. of London  |      |        |             | 1<br>2 |        |        | 1      | 1   | 1   | 1   |
|                   | Univ. Coll. London<br>Univ. Coll. Nottingham                               |      |        | 1           |        | 1      |        | 1      |     |     |     |
|                   | Oxford University<br>British Fish. Service                                 |      |        | 1           |        | 1<br>1 |        |        |     |     |     |
| Canada            | Acadia University N. S.<br>Univ. British Columbia                          | 1    |        | 1           |        |        |        |        |     |     |     |
|                   | Dalhousie University, N. S.<br>McGill University, Ont.                     | 2    |        | 1           | 2      | 1<br>3 | 1<br>2 | 3      | 1   |     | 1   |
|                   | Univ. of Manitoba<br>Univ. of Montreal                                     | 1    | 1      | 1           | 1      | 1      | 1      |        |     |     |     |
|                   | Memorial University, N. F.<br>Coll. Ste. Marie, Montreal                   |      | 1<br>1 |             |        |        |        |        |     |     |     |
|                   | Univ. of Toronto<br>Univ. West. Ontario<br>Royal Soc. Canada               | 6    | 7<br>1 | 6<br>1<br>1 | 3      | 5      | 4      | 5      | 4   | 4   | 1   |
| Far East          | Womens Medical College, Madras,<br>India                                   |      |        |             |        |        | 1      |        |     |     |     |
|                   | Judson Coll. Rangoon, Burma  |      |        |             |        |        | 1      |        |     |     |     |
| China<br>Colombia | Peking Union Med. Coll.<br>Cotton Res. Station                             |      |        |             |        |        | 1      |        |     |     | 1   |
| Denmark           | Carlsberg Laboratory<br>Univ. of Copenhagen                                |      | 1      |             |        |        |        | 1<br>1 | 1   |     |     |
| Egypt             | Egyptian Educ. Commission  |      | 1      |             |        |        |        |        |     |     |     |

EXHIBIT 4—*Continued*

|                  |  | 1934 | '35 | '36 | '37    | '38 | '39 | '40 | '41 | '42 | '43 |
|------------------|--|------|-----|-----|--------|-----|-----|-----|-----|-----|-----|
| France           | Pasteur Institute, Paris<br>University of Paris              |      |     |     | 1      | 1   |     |     |     |     |     |
|                  | University of Strasbourg<br>University of Strasbourg Medical |      |     |     | 1<br>1 |     |     |     |     |     |     |
| Germany          | University of Berlin<br>Neurolog. Inst. Frankfurt            |      |     | 1   | 1<br>2 | 1   |     |     |     |     |     |
|                  | University of Munich   |      |     | 1   |        |     |     |     |     |     |     |
| Hungary          | University of Debrescen<br>Budapest Univ. Medical            |      |     |     | 1      |     |     |     |     |     |     |
|                  | Franz Joseph University                                      |      | 1   |     |        | 1   |     |     |     |     |     |
| Italy            | University of Padua  |      | 1   |     | 1      |     |     |     |     |     |     |
| Japan            | Misaki Biolog. Inst.   |      |     | 1   |        |     |     |     |     |     |     |
| Norway           | University of Oslo   |      |     |     |        |     |     | 1   |     |     |     |
| Peru             | Guano Administration   |      |     |     |        |     |     |     |     | 1   |     |
| Poland           | University of Lwow   |      | 1   |     | 1      |     |     |     |     |     |     |
| Russia           | Moscow, Inst. Genetics                                       |      |     | 2   |        |     |     |     |     |     |     |
|                  | Belgrade Medical Coll.                                       |      | 1   |     | 1      |     |     |     |     |     |     |
| Spain            | Barcelona Medical Coll.                                      |      |     | 1   |        |     |     |     |     |     |     |
|                  | University of Lund   |      | 1   |     | 1      |     |     |     |     |     |     |
| Sweden           | Karolinska Inst. Stockholm<br>University of Stockholm        | 1    | 1   | 1   | 1      |     |     |     |     |     |     |
| Switzer-<br>land | Physiological Inst. Berne                                    | 1    |     |     |        |     |     |     |     |     |     |
|                  | Zoological Inst. Berne<br>University of Geneva               |      |     | 1   | 1      |     |     |     |     |     |     |
| Uruguay          | Ministry of Pub. Health                                      |      |     |     |        |     |     |     |     |     | 1   |
| Cuba             | University of Havana   |      |     |     |        | 2   | 2   |     |     |     |     |

*Summary*

|                               | 1923-33    | 1934-43    |
|-------------------------------|------------|------------|
| Universities and Colleges     | 246        | 269        |
| High Schools and Academies    | 30         | 50         |
| Institutes, Foundations, etc. | 56         | 31         |
| Foreign Institutions          | 101        | 59         |
|                               | <u>433</u> | <u>409</u> |

## EXHIBIT 5

## SUBSCRIBING AND COOPERATING INSTITUTIONS

A cooperating institution is one that has subscribed for the two preceding years, or that announces its intention of subscribing regularly. A subscribing institution is one that pays for one or more tables or rooms.

|                                | 1934 | '35 | '36 | '37 | '38 | '39 | '40 | '41 | '42 | '43 |
|--------------------------------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| American University            | x    |     | x   | x   | x   |     |     | x   |     |     |
| Amherst College                | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Atlanta University             | x    | x   | x   | x   |     | x   |     |     | x   | x   |
| Barnard College                | x    | x   |     |     | x   |     | x   | x   |     |     |
| Belgian-Amer. Educ. Found.     |      |     |     |     | x   | x   |     |     |     |     |
| Bell Telephone Laboratory      |      |     |     |     |     |     |     |     |     | x   |
| Berea College                  | x    | x   |     |     |     |     |     |     |     |     |
| Beth Israel Hospital           |      |     |     |     |     |     |     |     | x   |     |
| Biological Institute, Phila.   |      |     |     |     |     |     | x   | x   | x   | x   |
| Bowdoin College                | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Brooklyn College               |      |     |     | x   | x   | x   | x   | x   | x   | x   |
| Brown University               |      |     |     |     |     |     |     |     |     |     |
| Bryn Mawr College              | x    | x   | x   | x   | x   | x   | x   | x   | x   |     |
| Buffalo University Medical     |      |     |     |     |     | x   |     |     |     |     |
| Butler University              |      | x   |     |     |     |     |     |     |     |     |
| C. R. B. Educational Found.    |      |     |     | x   |     |     |     |     |     |     |
| California Inst. Technol.      |      | x   | x   | x   | x   | x   | x   | x   |     |     |
| Canisius College               |      |     |     |     |     | x   | x   | x   | x   |     |
| Carnegie Inst. Washington      |      |     |     |     | x   | x   |     |     |     |     |
| Catholic Univ. of America      |      |     |     |     |     |     |     |     |     | x   |
| Chicago, University of         | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Chicago, University of Medical |      | x   |     |     |     |     |     |     |     |     |
| Children's Hospital Cincinnati |      | x   |     |     |     |     |     |     |     |     |
| Chinese Educational Mission    | x    |     |     |     |     |     |     |     |     |     |
| Christ Hospital, Cincinnati    |      | x   |     |     |     |     |     |     |     |     |
| Cincinnati, University of      | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Columbia University            | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Columbia University Medical    |      |     |     | x   | x   | x   | x   | x   | x   | x   |
| Commonwealth Fund              | x    |     |     |     |     |     |     |     |     | x   |
| Connecticut College            |      |     |     |     | x   | x   |     |     |     |     |
| Cornell University             | x    | x   | x   | x   |     |     | x   | x   | x   | x   |
| Cornell University Medical     | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Dalhousie University           | x    |     |     |     | x   | x   |     |     |     |     |
| Dartmouth College              |      |     |     | x   | x   | x   |     |     |     |     |
| De Pauw University             | x    | x   | x   | x   | x   | x   | x   | x   |     |     |
| Drew University                |      |     |     |     | x   | x   |     |     | x   |     |

## EXHIBIT 5—Continued

|                                  | 1934 | '35 | '36 | '37 | '38 | '39 | '40 | '41 | '42 | '43 |
|----------------------------------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Duke University                  | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Eli Lilly Research Lab.          | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Elmira College                   |      |     |     |     | x   | x   |     |     |     |     |
| Fisk University                  |      |     |     |     |     |     | x   |     |     |     |
| Fordham University               |      |     |     |     |     |     |     | x   |     | x   |
| Frick Educational Comm.          |      |     |     |     |     |     |     |     | x   |     |
| General Education Board          | x    | x   | x   | x   | x   |     |     |     |     |     |
| Georgia, Univ. of Medical        |      |     |     | x   |     |     |     |     |     |     |
| Goucher College                  | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Hamilton College                 |      | x   | x   | x   |     | x   |     | x   |     |     |
| Harvard University               | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Harvard University Medical       | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Heidelberg College               |      |     |     |     |     |     |     |     | x   |     |
| Howard University                |      |     |     |     |     | x   |     |     |     |     |
| Hunter College                   | x    | x   | x   | x   | x   | x   | x   |     | x   |     |
| Illinois, University of          | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Indiana University               |      |     |     |     |     |     |     | x   |     |     |
| Industrial and Engin. Chem.      | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Iowa, State Univ. of             | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Iowa, State College of           | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Johns Hopkins University         | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Johns Hopkins University Medical | x    |     |     |     |     | x   | x   |     |     |     |
| Johnson Foundation               |      |     | x   |     |     |     |     |     |     | x   |
| Josiah Macy Foundation           |      |     |     |     |     |     |     | x   |     |     |
| Julius Rosenwald Fund            |      |     |     |     |     |     |     |     | x   |     |
| Kansas, University of            | x    |     | x   |     |     |     | x   |     |     |     |
| Kenyon College                   |      |     |     | x   | x   |     |     |     |     |     |
| Leland Stanford Univ.            |      |     |     |     |     |     | x   |     |     |     |
| Lincoln University               |      | x   |     |     |     |     |     |     |     |     |
| Long Island University           | x    | x   | x   | x   | x   | x   | x   |     | x   |     |
| Loyala Univ. Medical             |      |     |     |     |     |     |     | x   |     |     |
| McGill University                |      |     |     |     |     | x   |     |     |     |     |
| Markle Foundation                |      |     |     |     |     |     |     |     | x   |     |
| Maryland, Univ. of Medical       |      |     | x   | x   | x   | x   |     | x   | x   | x   |
| Marine Studios, Inc.             |      |     |     |     |     |     |     |     | x   |     |
| Mass. General Hospital           |      |     |     |     |     |     |     |     |     | x   |
| Mass. State College              |      |     |     | x   | x   | x   | x   | x   | x   |     |
| Memorial Hospital, N. Y.         |      | x   | x   | x   | x   | x   |     |     |     |     |
| Michigan, University of          |      |     |     |     |     |     |     | x   |     |     |
| Minnesota, University of         |      |     | x   | x   | x   |     |     |     |     |     |
| Missouri, University of          |      | x   | x   |     | x   | x   | x   | x   | x   |     |
| Morehouse College                | x    | x   | x   | x   |     | x   |     |     | x   |     |

|  | 1934        | '35         | '36         | '37         | '38         | '39         | '40         | '41         | '42         | '43         |
|--|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Mt. Holyoke College<br>Mt. Sinai Hospital<br>Mundelein University                                | x           | x           | x           | x           | x           | x           | x           | x           | x           | x           |
| National Research Council<br>N. Y. Dept. of Health<br>New York University                        | x<br>x      | x<br>x      | x<br>x      | x<br>x      | x<br>x      | x<br>x      | x<br>x      | x<br>x      | x<br>x      | x<br>x      |
| New York University Medical<br>New York University Wash. Square<br>N. Carolina Coll. for Negroes | x<br>x      | x<br>x      | x<br>x      | x<br>x      | x<br>x      | <br>x       | x<br>x      | x<br>x      | x<br>x      | x<br>x      |
| Northwestern University<br>Notre Dame University<br>Oberlin College                              | x<br><br>x  | x<br><br>x  | x<br><br>x  | x<br><br>x  | x<br><br>x  | x<br><br>x  | x<br><br>x  | x<br><br>x  | <br><br>x   | <br><br>x   |
| Ohio State University<br>Ohio Wesleyan University<br>Penn. College for Women                     | <br><br>x   | <br><br>x   | <br><br>x   | <br><br>x   | <br><br>x   | <br><br>x   | x<br><br>x  | x<br><br>x  | x<br><br>x  | x<br><br>x  |
| Pennsylvania, Univ. of<br>Pennsylvania, Univ. of Medical<br>Pittsburgh, Univ. of                 | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x |
| Princeton University<br>Purdue University<br>Radcliffe College                                   | x<br><br>x  | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x | x<br>x<br>x | x<br><br>x  |
| Rensselaer Poly. Inst.<br>Rice Institute<br>Rochester, University of                             | <br><br>x   | <br><br>x   | <br>x<br>x  | <br>x<br>x  | <br><br>x   | <br><br>x   | <br><br>x   | <br><br>x   | <br><br>x   | <br><br>x   |
| Rochester, University of Medical<br>Rockefeller Foundation<br>Rockefeller Institute              | x<br>x<br>x | x<br>x<br>x | <br>x<br>x  | x<br>x<br>x | x<br>x<br>x | <br>x<br>x  | <br>x<br>x  | <br>x<br>x  | <br>x<br>x  | <br><br>x   |
| Royal Egyptian Foundation<br>Russell Sage College<br>Rutgers University                          | <br><br>x   | x<br><br>   | <br>x<br>x  | <br>x<br>x  | <br>x<br>x  | <br>x<br>x  | <br>x<br>x  | <br>x<br>x  | <br>x<br>x  | <br><br>x   |
| St. Elizabeth, College of<br>St. Francis Xavier College<br>St. Johns College                     | x<br><br>   | <br><br>x   | <br>x<br>x  | <br>x<br>x  | <br>x<br>x  | <br>x<br>x  | <br><br>    | <br><br>    | <br><br>    | <br><br>    |
| Sarah Lawrence College<br>Seton Hall College<br>Seton Hill College                               | <br><br>    | <br>x<br>x  | <br>x<br>x  | <br><br>x   | <br><br>    | <br><br>    | <br><br>    | x<br><br>x  | <br><br>    | <br><br>x   |
| Smith College<br>J. C. Smith University<br>Spring Hill College                                   | x<br>x      | x<br>x      | x<br><br>   | x<br><br>   | x<br><br>x  | x<br><br>   | x<br><br>   | x<br><br>   | x<br><br>   | x<br><br>   |
| Springfield College<br>Swarthmore College<br>Sweet Briar College                                 | x<br><br>   | x<br><br>   | x<br><br>   | x<br><br>   | x<br><br>   | <br><br>    | x<br><br>   | x<br><br>   | <br><br>x   | <br><br>    |

EXHIBIT 5—*Continued*

|                                 | 1934 | '35 | '36 | '37 | '38 | '39 | '40 | '41 | '42 | '43 |
|---------------------------------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Syracuse University             | x    | x   | x   | x   | x   |     | x   | x   | x   |     |
| Syracuse University Medical     |      |     |     |     |     | x   | x   |     |     |     |
| Temple University               | x    | x   | x   | x   | x   | x   |     |     |     |     |
| Toledo, University of           |      |     |     |     | x   | x   |     | x   |     |     |
| Tufts College                   | x    | x   | x   |     | x   | x   | x   | x   | x   | x   |
| Tulane University               |      |     |     |     |     |     |     | x   |     |     |
| Tulane University Newcomb Coll. | x    |     |     |     |     |     |     | x   |     |     |
| Union College, N. Y.            |      | x   | x   | x   | x   |     |     | x   | x   |     |
| Union College, Ky.              |      |     |     |     |     | x   | x   |     |     |     |
| U. S. Fish and Wild Life Serv.  |      |     |     |     |     |     |     |     |     | x   |
| Vanderbilt University           |      | x   |     |     |     |     |     |     |     |     |
| Vanderbilt University Medical   | x    | x   | x   | x   | x   | x   | x   | x   | x   |     |
| Vassar College                  | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Vermont, University of          |      | x   |     |     | x   |     |     |     |     |     |
| Villanova College               |      |     |     |     |     | x   | x   | x   | x   |     |
| Virginia, University of         | x    | x   | x   | x   | x   | x   | x   | x   |     |     |
| Wabash College                  | x    | x   | x   | x   | x   |     | x   | x   |     |     |
| Washington University           |      |     |     | x   | x   | x   | x   | x   | x   | x   |
| Washington University Medical   |      |     |     | x   | x   | x   | x   | x   |     |     |
| Wellesley College               | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Wesleyan University             | x    | x   | x   | x   | x   | x   | x   | x   | x   |     |
| Western Reserve University      |      | x   |     |     | x   | x   |     |     |     |     |
| Wheaton College                 | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| William and Mary College        |      |     |     |     | x   | x   |     |     |     |     |
| Williams College                |      |     |     |     | x   | x   |     |     |     |     |
| Wilson College                  | x    | x   | x   | x   | x   | x   | x   |     |     |     |
| Wisconsin, University of        |      | x   | x   | x   |     |     |     |     |     |     |
| Wistar Institute                | x    | x   | x   | x   |     |     |     |     |     |     |
| Woods Hole Oceanographic Inst.  |      |     |     |     |     |     |     |     | x   | x   |
| Yale University                 | x    | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| Yale University Medical         |      |     |     |     |     |     | x   | x   |     |     |

# EXHIBIT 6

## ADDITIONS TO THE LIBRARY

|                   | Bound<br>volumes | Reprints | Sets<br>completed | Partially<br>completed | New<br>journals | Classics | Budget   |
|-------------------|------------------|----------|-------------------|------------------------|-----------------|----------|----------|
| 1934              | 1,138            | 5,028    | 8                 | 16                     | 18              |          | \$20.325 |
| 1935              | 1,622            | 4,478    | 24                | 17                     | 55              |          | 22.444   |
| 1936              | 2,107            | 3,339    | 29                | 12                     | 28              |          | 22.510   |
| 1937              | 1,155            | 7,042    | 24                | 20                     | 21              |          | 22.029   |
| 1938              | 1,455            | 6,905    | 33                | 30                     | 15              |          | 19.515   |
| 1939              | 1,239            | 3,850    | 33                | 24                     | 21              |          | 22.149   |
| 1940              | 1,561            | 3,528    | 20                | 46                     | 21              |          | 17.923   |
| 1941              | 1,482            | 3,321    | 24                | 91                     | 25              | 4        | 16.964   |
| 1942              | 1,758            | 3,097    | 45                | 161                    | 13              | 23       | 15.332   |
| 1943              | 1,008            | 7,927    | 10                | 51                     | 10              | 5        | 11.047   |
| Total added       | 14,525           | 48,515   | 250               | 468                    | 227             | 32       |          |
| Total in<br>Libr. | 51,945           | 129,723  | 1800<br>approx.   | 600<br>approx.         |                 |          |          |

### 4. THE STAFF, 1944

CHARLES PACKARD, Director, Marine Biological Laboratory, Woods Hole, Massachusetts.

#### SENIOR STAFF OF INVESTIGATION

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 MADELENE E. PIERCE, Assistant Professor of Zoology, Vassar College.  
 W. M. REID, Assistant Professor of Biology, Monmouth College.  
 MARY D. ROGICK, Professor of Biology, College of New Rochelle.

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MARY E. BANKS, Washington University.

## EMBRYOLOGY

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H. B. GOODRICH, Professor of Biology, Wesleyan University.

## II. INSTRUCTORS

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WALTER E. GARREY, Professor of Physiology, Vanderbilt University Medical School.  
MERKEL H. JACOBS, Professor of Physiology, University of Pennsylvania.

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ROBERT BALLENTINE, Lecturer in Zoology, Columbia University.  
ARTHUR C. GIESE, Associate Professor of Biology, Stanford University (absent in 1943).  
RUDOLF T. KEMPTON, Professor of Zoology, Vassar College.

## BOTANY

## I. CONSULTANTS

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B. M. DUGGAR, Professor of Plant Physiology, University of Wisconsin.

## II. INSTRUCTORS

WM. RANDOLPH TAYLOR, Professor of Botany, University of Michigan, in charge of course.  
HANNAH CROASDALE, Technical Assistant, Dartmouth College.

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L. ROBINSON HYDE, Phillips Exeter Academy, Exeter, N. H.

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## Independent Investigators, 1944

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SCHNEYER, LEON H., Instructor, New York University College of Dentistry.  
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CHOUKROUN, NINE, Cornell University Medical College.  
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COX, EDWARD H., Professor of Chemistry, Swarthmore College.  
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SPEIDEL, CARL C., Professor of Anatomy, University of Virginia.  
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TASHIRO, KIYOSHI, University of Cincinnati, College of Medicine.  
WAGNER, CARROLL E., Research Assistant, Histology, Naval Medical Research Institute.  
WEIDENREICH, FRANZ, Honorary Director, Cenozoic Research Laboratory, China.  
WEINER, NATHAN, Director of Research, Endo Products, Inc.

WILLIER, B. H., Professor of Zoology, Director of Biological Laboratories, The Johns Hopkins University.

WOODWARD, ALVALYN E., Assistant Professor, University of Michigan.

ZORZOLI, ANITA, Teaching Fellow, New York University.

#### Research Assistants, 1944

ABRAMSKY, TESSIE, Technician, Rockefeller Institute for Medical Research.

BRUNELLI, ELEANOR L., Research Assistant, New York University Dental School.

DEFALCO, ROSE H., Research Assistant-Secretary, University of Pennsylvania.

DZIORNEY, LEON, Research Assistant, New York University.

FRUMIN, M. R., Research Assistant, University of Pennsylvania.

GOLDIS, BERNICE R., Research Assistant, University of Pennsylvania.

GREGG, JOHN R., Graduate Student, Princeton University.

HIRST, SHIRLEY M., Research Assistant, University of Pennsylvania.

HONEGGER, CAROL, Temple University.

HOPKINS, AMOS, Junior Engineering Aide, Massachusetts State Health Department.

LAWLER, H. CLAIRE, Research Assistant, New York University.

LEFEVRE, LINDA, Research Assistant, University of Pennsylvania.

LEFEVRE, PAUL G., Research Assistant, University of Pennsylvania.

LEVY, BETTY, Laboratory Technician, Rockefeller Institute.

MARKS, MILDRED H., Assistant Research Worker, University of Pennsylvania.

MORTON, JANE W., Technical Assistant in Zoology, University of Pennsylvania.

PRICE, WINSTON HARVEY, Research Assistant, University of Pennsylvania.

QUINN, GERTRUDE P., Research Assistant, New York University.

WILSON, WALTER L., Research Associate, University of Pennsylvania.

WOODWARD, ARTHUR A., Research Assistant, University of Pennsylvania.

#### Students, 1944

##### BOTANY

CHEW, ROBERT M., Student, Washington & Jefferson College.

DEVINE, VERONA, Student, Hunter College.

GUZMAN, JULIA, Student, Washington University.

HOSKINS, BARBARA, Student, Wellesley College.

MITTLACHER, HELEN, Student, Wheaton College.

##### EMBRYOLOGY

ANDERSON, JOAN C., Student, McGill University.

COURANT, GERTRUDE E., Student, Swarthmore, College.

CULLEN, SISTER MARY URBAN, Graduate Student, Yale University.

DAVIDSON, MARGARET E. M., Student, McGill University.

FARFANTE, ISABEL PEREZ, Student, Cambridge, Massachusetts.

FINKELSTEIN, GRACE, Teaching Fellowship, New York University.

GETZ, CHARLOTTE E., Undergraduate Student, University of Chicago.

GODWIN, DORIS RUTH, Graduate Assistant, University of North Carolina.

HENLEY, CATHERINE, Graduate Assistant, University of North Carolina.

HONEGGER, CAROL MARIE, Student, Temple University.

KELLEY, ELLEN MARY, Student, New Jersey College for Women.

KIVY, EVELYN, Instructional Staff, Brooklyn College.

LANDAU, CAROL, Student, Goucher College.

LANTZ, ELSIE JEAN, Student, Washington University.

McGOVERN, BEULAH H., Teaching Fellow, New York University.

MURRAY, HELEN ERNESTINE, Student, Emmanuel College.

POTTS, ELLA ELIZABETH, Student, Sarah Lawrence College.

ROTH, OWEN H., Instructor in Biology, St. Vincent College.

SCHNELLER, SISTER MARY BEATRICE, Professor, Saint Joseph College for Women.  
STRONG, HELEN MARGARET, Teaching Fellow, Smith College.  
VISHNIAC, WOLF, Student, Brooklyn College.  
WILLIAMSON, FRANCES ALICE, Student, New Jersey College for Women.  
WILLIS, MARIAN, Student, Iowa State College.

## PHYSIOLOGY

BERNSTEIN, JEANE, Graduate Student, New York University.  
CARSON, GWENETH, Student, University of Toronto.  
CREGAR, MARY, Demonstrator in Physiology, Bryn Mawr College.  
KEISTER, MARGARET LOUISE, Instructor in Zoology, Wheaton College.  
MCLEAN, DOROTHY JUANITA, Graduate Student, University of Toronto.  
PARTRIDGE, JUDITH ANN, Assistant in Physiology, Vassar College.  
PEPPER, BILLIE BARBARA, Student, Radcliffe College.  
REICH, EVA, Student, Barnard College.  
TAYLOR, BABETTE, Student, Washington University.  
THERIEN, MERCEDES, Assistant Research, Montreal University.

## ZOOLOGY

AUSTIN, JANE, Student, Randolph-Macon Woman's College.  
BANKS, MARY ELIZABETH, Research Assistant, Washington University.  
BARROWS, SHIRLEY LOUISE, Student, University of Rochester.  
BENSON, ELEANORE BIE, Student, University of Pennsylvania.  
BUTT, FERDINAND H., Instructor, Cornell University.  
CALKINS, JANET ELIZABETH MORSE, University of Chicago.  
CONRAVEY, JUNE ROSE, Student Assistant, Newcomb College, Tulane University.  
DEREVERE, JOAN BROOKS, Undergraduate Student, Wilson College.  
DOUGLIS, MARJORIE B., Assistant in Zoology, Chicago University.  
DUNBAR, SALLY, De Pauw University.  
FAIRFIELD, JANET, Student, Russell Sage College.  
FALKNER, ETTA, Instructor, American Museum of Natural History.  
FOGERSON, VIRGINIA LEE, Drury College.  
GOSFORD, BARBARA, Duke University.  
HABERT, YVONNE A., High School Teacher, City of Boston.  
KOOPMAN, KARL FRIEDRICH, Graduate Student, Columbia University.  
LANGMAN, IDA K., University of Pennsylvania.  
LAUTHERS, ROSEMARY ANN, Student, Oberlin College.  
LEDUC, ELIZABETH HORTENSE, Graduate Assistant, Wellesley College.  
LLOYD, MARY REMSEN, Vassar College.  
MARKS, MILDRED HELEN, Graduate Student, University of Pennsylvania.  
MCCLINTOCK, MARY, Instructor, Bemidji, Minnesota.  
NEAL, LUCY LEE, Drury College.  
RANDALL, NANCY LOIS, Student, Swarthmore College.  
REESE, JEAN, Goucher College.  
ROOT, OSCAR M., Instructor, Brooks School.  
ROTH, OWEN HAROLD, Instructor in Biology, St. Vincent College.  
SCHMID, LEO A., Baltimore, Md.  
SLAVIN, ALICE CECILIA, Student, Seton Hill College.  
SOUTHWELL, VIOLET M., Student, Wilson College.  
STEENBURG, ISABELLA, Student, Vassar College.  
STEKL, ELEANOR B., Science Teacher, N. Tonawanda High School.  
SWEENEY, PATRICIA GEORGIA, Student, Oberlin College.  
VAN GEYT, VIRGINIA, Student, University of Rochester.  
VIOSCA, MIRIAM A., Student Assistant, Newcomb College, Tulane University.  
WELLER, DORIS A., Undergraduate, Radcliffe College.  
WARNER, ROSE ELLA, Teacher of Biology, Frick Educational Commission.

## 6. TABULAR VIEW OF ATTENDANCE

|   | 1940 | 1941 | 1942 | 1943 | 1944 |
|---|------|------|------|------|------|
| INVESTIGATORS—Total.....  | 386  | 337  | 201  | 160  | 193  |
| Independent.....  | 253  | 197  | 132  | 89   | 112  |
| Under instruction.....  | 62   | 59   | 16   | 19   | 11   |
| Library readers.....  | —    | 31   | 28   | 35   | 50   |
| Research assistants.....  | 71   | 50   | 25   | 17   | 20   |
| STUDENTS—Total.....   | 128  | 131  | 74   | 68   | 75   |
| Zoology.....  | 55   | 55   | 36   | 47   | 37   |
| Protozoology (not given after 1940).....                        | 7    | —    | —    | —    | —    |
| Embryology.....   | 34   | 37   | 24   | 13   | 23   |
| Physiology.....   | 22   | 24   | 6    | 8    | 10   |
| Botany.....   | 10   | 15   | 8    | —    | 5    |
| TOTAL ATTENDANCE.....   | 514  | 468  | 275  | 228  | 276  |
| Less persons registered as both students and investigators..... | 7    | 7    | 2    | 6    | 1    |
|   | 507  | 461  | 273  | 222  | 275  |
| INSTITUTIONS REPRESENTED—Total.....                             | 148  | 144  | 126  | 116  | 106  |
| By investigators.....   | 112  | 102  | 83   | 70   | 74   |
| By students.....  | 79   | 72   | 43   | 41   | 41   |
| SCHOOLS AND ACADEMIES REPRESENTED.....                          |      |      |      |      |      |
| By investigators.....   | 1    | 5    | 2    | 2    | 1    |
| By students.....  | 2    | 2    | —    | 1    | 2    |
| FOREIGN INSTITUTIONS REPRESENTED.....                           |      |      |      |      |      |
| By investigators.....   | 2    | 3    | —    | 2    | 2    |
| By students.....  | 1    | 1    | —    | —    | 3    |

## 7. SUBSCRIBING AND COOPERATING INSTITUTIONS

1944

|  |   |
|--|---|
| Amherst College  | H. Sophie Newcomb College                     |
| Barnard College  | New York University                           |
| Bowdoin College  | New York University College of Medicine       |
| Brooklyn College   | New York University Washington Square College |
| Bryn Mawr College  | Oberlin College                               |
| Catholic University of America   | Ohio State University                         |
| Columbia University  | Princeton University                          |
| Cornell University   | Radcliffe College                             |
| Cornell University Medical College                                     | Rockefeller Institute for Medical Research    |
| Duke University  | Russell Sage College                          |
| Fish and Wild Life Service, U. S. Dept. of the Interior                | St. Joseph College for Women                  |
| Fordham University   | Smith College                                 |
| Henry C. Frick Educational Commission                                  | State University of Iowa                      |
| Goucher College  | Syracuse University                           |
| Harvard University   | Syracuse University Medical School            |
| Hunter College   | Temple University                             |
| Industrial and Engineering Chemistry, of the American Chemical Society | Tufts College                                 |
| Johns Hopkins University   | University of Chicago                         |
| The Lankenau Hospital Research Institute                               | University of Cincinnati                      |
| Eli Lilly and Co.  | University of Illinois                        |
| Massachusetts Department of Health                                     | University of Maryland Medical School         |
| Massachusetts Institute of Technology                                  | University of Pennsylvania                    |
| Mount Holyoke College  | University of Pennsylvania School of Medicine |
|  | University of Rochester                       |

Vassar College  
 Villanova College  
 Washington University  
 Wayne University  
 Wellesley College  
 Wesleyan University

Western Reserve University  
 Wheaton College  
 Wilson College  
 Wistar Institute  
 Woods Hole Oceanographic Institution  
 Yale University

## 8. EVENING LECTURES, 1944

Friday, June 30

DR. T. H. BISSENETTE ..... "Some Recent Studies on Photoperiodicity  
 in Animals, particularly Fur-bearers."

Friday, July 7

DR. ETHEL BROWNE HARVEY ..... "Some Results of Centrifuging the Arbacia  
 Egg."

Friday, July 14

DR. A. C. GIESE ..... "Ultraviolet Radiations and the Life Activi-  
 ties of Cells."

Friday, July 21

DR. H. J. MULLER ..... "Evidence for the Meticulousness of Adap-  
 tation."

Friday, July 28

DR. CARL C. SPEIDEL ..... "Experimental Studies of Special Sensory  
 Organs and Nerves."

Thursday, August 3

DR. ERNEST CARROLL FAUST ..... "Problems of Tropical Medicine in the  
 United States."

Friday, August 4

DR. A. K. PARPART ..... "Blood Preservation: A Problem in Cellu-  
 lar Physiology."

Thursday, August 10

MR. G. G. LOWER ..... "Local Invertebrates."

Friday, August 11

DR. W. C. ALLEE ..... "Social Orders Among Vertebrates."

Wednesday, August 16

DR. RALPH TURNER ..... "Rehabilitation of Scientific Institutions in  
 Devastated Europe."

Friday, August 18

DR. A. W. POLLISTER ..... "The Centriole Problem."

Friday, August 25

PROF. G. H. PARKER ..... "Animal Coloration, Fixed and Changeable."

## 9. SHORTER SCIENTIFIC PAPERS, 1944

Tuesday, July 18

DR. B. H. WILLIER ..... "Melanophore Control of Sexual Dimor-  
 phism in Feather Pigmentation of the  
 Barred Rock Fowl."

DR. VIKTOR HAMBURGER ..... "The Effects of Peripheral Factors on  
 Motor Neuron Differentiation in the Chick  
 Embryo."

DR. W. H. LEWIS ..... "The Superficial Gel Layer and Its Role in  
 Development."

## Tuesday, July 25

- DR. LEONOR MICHAELIS ..... "Ferritin and Iron Metabolism."  
 DR. ARNOLD LAZAROW ..... "The Chemical Organization of the Cytoplasm of the Liver Cell."  
 DR. LEONOR MICHAELIS ..... "Theory of Metachromatic Staining."

## Tuesday, August 1

- DR. DOROTHY WRINCH ..... "The Native Protein in Crystalline Form."  
 DR. OTTO MYERHOF ..... "The Role of Adenylpyro-Phosphatase in Alcoholic Fermentation of Yeast."  
 DR. ERNEST SCHARRER ..... "The Naples Station Still Lives."

## Tuesday, August 8

- DR. A. M. SHANES ..... "Application of Bio-electricity to the Study of Functioning in Nerve."  
 DR. DAVID NACHMANSOHN ..... "On the Energy Source of the Nerve Action Potential."  
 DR. T. H. BULLOCK ..... "Oscillographic Studies on the Giant Nerve Fiber System in Lumbricus."  
 DR. PAUL WEISS ..... "Evidence for the Perpetual Proximo-distal Growth of Nerve Fibers."

## Tuesday, August 15

- DR. L. V. HEILBRUNN ..... "A Toxic Substance from Protoplasm."  
 DR. D. L. HARRIS .....  
 DR. P. G. LEFEVRE .....  
 DR. W. H. PRICE .....  
 DR. W. L. WILSON .....  
 DR. A. A. WOODWARD, JR. ....  
 DR. D. L. HARRIS ..... "The Chemical Nature of a Toxic Substance from Protoplasm."  
 DR. W. H. PRICE .....  
 DR. L. V. HEILBRUNN .....  
 DR. G. I. LAVIN ..... "Recent Developments in Ultraviolet Microscopy."

## Tuesday, August 22

- DR. B. W. ZWEIFACH ..... "The Peripheral Circulation in Traumatic Shock."  
 DR. W. R. AMBERSON ..... "Recent Experience with Hemoglobin-saline Solutions."  
 DR. R. G. ABELL ..... "Gelatin as a Plasma Substitute."  
 DR. W. M. PARKINS .....

## Thursday, August 24

- DR. C. A. BERGER ..... "Experimental Studies on the Cytology of Allium."  
 DR. VALY MENKIN ..... "Studies on the Chemical Basis of Fever."  
 DR. MIRIAM F. MENKIN ..... "In Vitro Fertilization of Human Ova."

## Tuesday, August 29

- DR. L. M. BERTHOLF ..... "Studies on Metamorphosis in the Tunicate."  
 E. MORTENSEN ..... "Behavior and Tube Building Habits of Polydora ligni."  
 DR. P. S. GALTSOFF .....  
 DR. J. B. BUCK ..... "The Click Mechanism of Elaterid Beetles."

Thursday, August 31

- DR. G. K. SMELSER ..... "Orbital Changes in Experimental Exophthalmos."  
 DR. A. GOREMAN ..... "Radioactive Iodine Absorption in Lower Chordates and the Problem of Homology of the Thyroid Gland."  
 DR. D. L. HARRIS ..... "Phosphoprotein Phosphatase, a New Enzyme from the Frog Egg."

## 10. MEMBERS OF THE CORPORATION, 1944

### 1. LIFE MEMBERS

- ALLIS, MR. E. P., JR., Palais Carnoles, Menton, France.  
 ANDREWS, MRS. GWENDOLEN FOULKE, Baltimore, Maryland.  
 BECKWITH, DR. CORA J., Vassar College, Poughkeepsie, New York.  
 BILLINGS, MR. R. C., 66 Franklin Street, Boston, Massachusetts.  
 CALVERT, DR. PHILIP P., University of Pennsylvania, Philadelphia, Pennsylvania.  
 COLE, DR. LEON J., College of Agriculture, Madison, Wisconsin.  
 CONKLIN, PROF. EDWIN G., Princeton University, Princeton, New Jersey.  
 COWDRY, DR. E. V., Washington University, St. Louis, Missouri.  
 EVANS, MRS. GLENDOWER, 12 Otis Place, Boston, Massachusetts.  
 FOOT, MISS KATHERINE, Care of Morgan Harjes Cie, Paris, France.  
 JACKSON, MR. CHAS. C., 24 Congress Street, Boston, Massachusetts.  
 JACKSON, MISS M. C., 88 Marlboro Street, Boston, Massachusetts.  
 KING, MR. CHAS. A.  
 KINGSBURY, PROF. B. F., Cornell University, Ithaca, New York.  
 LEWIS, PROF. W. H., Johns Hopkins University, Baltimore, Maryland.  
 MEANS, DR. J. H., 15 Chestnut Street, Boston, Massachusetts.  
 MOORE, DR. GEORGE T., Missouri Botanical Gardens, St. Louis, Missouri.  
 MOORE, DR. J. PERCY, University of Pennsylvania, Philadelphia, Pa.  
 MORGAN, MRS. T. H., Pasadena, California.  
 MORGAN, PROF. T. H., Director of Biological Laboratory, California Institute of Technology, Pasadena, California.  
 NOYES, MISS EVA J.  
 PORTER, DR. H. C., University of Pennsylvania, Philadelphia, Pennsylvania.  
 SCOTT, DR. ERNEST L., Columbia University, New York City, New York.  
 SEARS, DR. HENRY F., 86 Beacon Street, Boston, Massachusetts.  
 SHEDD, MR. E. A.  
 THORNDIKE, DR. EDWARD L., Teachers College, Columbia University, New York City, New York.  
 TREADWELL, PROF. A. L., Vassar College, Poughkeepsie, New York.  
 TRELEASE, PROF. WILLIAM, University of Illinois, Urbana, Illinois.  
 WAITE, PROF. F. C., 144 Locust Street, Dover, New Hampshire.  
 WALLACE, LOUISE B., 359 Lytton Avenue, Palo Alto, California.

### 2. REGULAR MEMBERS

- ADAMS, DR. A. ELIZABETH, Mount Holyoke College, South Hadley, Massachusetts.  
 ADDISON, DR. W. H. F., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.

- ADOLPH, DR. EDWARD F., University of Rochester Medical School, Rochester, New York.
- ALBAUM, DR. HARRY G., 3115 Avenue I, Brooklyn, New York.
- ALBERT, DR. ALEXANDER, Biological Laboratories, Harvard University, Cambridge, Massachusetts.
- ALLEE, DR. W. C., The University of Chicago, Chicago, Illinois.
- AMBERSON, DR. WILLIAM R., Department of Physiology, University of Maryland, School of Medicine, Lombard and Greene Streets, Baltimore, Maryland.
- ANDERSON, DR. RUBERT S., University of Maryland School of Medicine, Department of Physiology, Baltimore, Maryland.
- ANDERSON, DR. T. F., University of Pennsylvania, Philadelphia, Pennsylvania.
- ARMSTRONG, DR. PHILIP B., College of Medicine, Syracuse University, Syracuse, New York.
- AUSTIN, DR. MARY L., Wellesley College, Wellesley, Massachusetts.
- BAITSELL, DR. GEORGE A., Yale University, New Haven, Connecticut.
- BAKER, DR. H. B., Zoological Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania.
- BALLARD, DR. WILLIAM W., Dartmouth College, Hanover, New Hampshire.
- BALLENTINE, DR. ROBERT, Columbia University, Department of Zoology, New York City, New York.
- BALL, DR. ERIC G., Department of Biological Chemistry, Harvard University Medical School, Boston, Massachusetts.
- BARD, PROF. PHILIP, Johns Hopkins Medical School, Baltimore, Maryland.
- BARRON, DR. E. S. GUZMAN, Department of Medicine, The University of Chicago, Chicago, Illinois.
- BARTH, DR. L. G., Department of Zoology, Columbia University, New York City, New York.
- BARTLETT, DR. JAMES H., Department of Physics, University of Illinois, Urbana, Illinois.
- BEADLE, DR. G. W., School of Biological Sciences, Stanford University, California.
- BEAMS, DR. HAROLD W., Department of Zoology, State University of Iowa, Iowa City, Iowa.
- BECK, DR. L. V., Hahnemann Medical College, Philadelphia, Pennsylvania.
- BEHRE, DR. ELINOR H., Louisiana State University, Baton Rouge, Louisiana.
- BERTHOLF, DR. LLOYD M., Western Maryland College, Westminster, Maryland.
- BIGELOW, DR. H. B., Museum of Comparative Zoology, Cambridge, Massachusetts.
- BIGELOW, PROF. R. P., Massachusetts Institute of Technology, Cambridge, Massachusetts.
- BINFORD, PROF. RAYMOND, Guilford College, North Carolina.
- BISSONNETTE, DR. T. HUME, Trinity College, Hartford, Connecticut.
- BLANCHARD, PROF. K. C., Johns Hopkins Medical School, Baltimore, Maryland.
- BODINE, DR. J. H., Department of Zoology, State University of Iowa, Iowa City, Iowa.
- BORING, DR. ALICE M., Yenching University, Peking, China.
- BRADLEY, PROF. HAROLD C., University of Wisconsin, Madison, Wisconsin.
- BRODIE, MR. DONALD M., 522 Fifth Avenue, New York City, New York.
- BRONFENBRENNER, DR. JACQUES J., Department of Bacteriology, Washington University Medical School, St. Louis, Missouri.

- BROOKS, DR. MATILDA M., University of California, Department of Zoology, Berkeley, California.
- BROOKS, DR. S. C., University of California, Berkeley, California.
- BROWN, DR. DUGALD E. S., New York University, College of Dentistry, 209 East 23d Street, New York City, New York.
- BROWN, DR. FRANK A., JR., Department of Zoology, Northwestern University, Evanston, Illinois.
- BUCKINGHAM, MISS EDITH N., Sudbury, Massachusetts.
- BUCK, DR. JOHN B., Department of Zoology, University of Rochester, Rochester, New York.
- BUDINGTON, PROF. R. A., Winter Park, Florida.
- BULLINGTON, DR. W. E., Randolph-Macon College, Ashland, Virginia.
- BURBANCK, DR. WILLIAM D., Department of Biology, Drury College, Springfield, Missouri.
- BURKENROAD, DR. M. D., Yale University, New Haven, Connecticut.
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- CANNAN, PROF. R. K., New York University College of Medicine, 477 First Avenue, New York City, New York.
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- CAROTHERS, DR. E. ELEANOR, 134 Avenue C. East, Kingman, Kansas.
- CARPENTER, DR. RUSSELL L., Tufts College, Tufts College, Massachusetts.
- CARROLL, PROF. MITCHELL, Franklin and Marshall College, Lancaster, Pennsylvania.
- CARVER, PROF. GAIL L., Mercer University, Macon, Georgia.
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- CATTELL, MR. WARE, 3609 Military Road, N. W., Washington, D. C.
- CHAMBERS, DR. ROBERT, Washington Square College, New York University, Washington Square, New York City, New York.
- CHASE, DR. AURIN M., Princeton University, Princeton, New Jersey.
- CHENEY, DR. RALPH H., Biology Department, Long Island University, Brooklyn, New York.
- CHIDESTER, PROF. F. E., Auburndale, Massachusetts.
- CHILD, PROF. C. M., Jordan Hall, Stanford University, California.
- CHURNEY, LT. LEON, 28th Alt. Tng. Unit, HAAF, Harlingen, Texas.
- CLAFF, MR. C. LLOYD, Department of Biology, Brown University, Providence, Rhode Island.
- CLARK, PROF. E. R., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.
- CLARK, DR. LEONARD B., Department of Biology, Union College, Schenectady, New York.
- CLARKE, DR. G. L., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts.
- CLELAND, PROF. RALPH E., Indiana University, Bloomington, Indiana.
- CLOWES, DR. G. H. A., Eli Lilly and Company, Indianapolis, Indiana.
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- COLLETT, DR. MARY E., Western Reserve University, Cleveland, Ohio.
- COLTON, PROF. H. S., Box 601, Flagstaff, Arizona.
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- COPELAND, PROF. MANTON, Bowdoin College, Brunswick, Maine.
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- COSTELLO, DR. HELEN MILLER, Department of Zoology, University of North Carolina, Chapel Hill, North Carolina.
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- CRANE, JOHN O., Woods Hole, Massachusetts.
- CRANE, MRS. W. MURRAY, Woods Hole, Massachusetts.
- CROASDALE, HANNAH T., Dartmouth College, Hanover, New Hampshire.
- CROWELL, DR. P. S., JR., Department of Zoology, Miami University, Oxford, Ohio.
- CURTIS, DR. MAYNIE R., 377 Dexter Trail, Mason, Michigan.
- CURTIS, PROF. W. C., University of Missouri, Columbia, Missouri.
- DAN, DR. KATSUMA, Misaki Biological Station, Misaki, Japan.
- DAVIS, DR. DONALD W., College of William and Mary, Williamsburg, Virginia.
- DAWSON, DR. A. B., Harvard University, Cambridge, Massachusetts.
- DAWSON, DR. J. A., The College of the City of New York, New York City, New York.
- DEDERER, DR. PAULINE H., Connecticut College, New London, Connecticut.
- DEMEREK, DR. M., Carnegie Institution of Washington, Cold Spring Harbor, Long Island, New York.
- DILLER, DR. WILLIAM F., 1016 South 45th Street, Philadelphia, Pennsylvania.
- DODDS, PROF. G. S., Medical School, University of West Virginia, Morgantown, West Virginia.
- DOLLEY, PROF. WILLIAM L., University of Buffalo, Buffalo, New York.
- DONALDSON, DR. JOHN C., University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania.
- DUBOIS, DR. EUGENE F., Cornell University Medical College, 1300 York Avenue, New York City, New York.
- DUGGAR, DR. BENJAMIN M., c/o Lederle Laboratories Inc., Pearl River, New York.
- DUNGAY, DR. NEIL S., Carleton College, Northfield, Minnesota.
- DURYEE, DR. WILLIAM R., Surgeon General's Office, Washington, D. C.
- EDWARDS, DR. D. J., Cornell University Medical College, 1300 York Avenue, New York City, New York.
- ELLIS, DR. F. W., Monson, Massachusetts.
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- FAURÉ-FREMIET, PROF. EMMANUEL, Collège de France, Paris, France.
- FAUST, DR. ERNEST C., Tulane University of Louisiana, New Orleans, Louisiana.
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- FISHER, DR. JEANNE M., Department of Biochemistry, University of Toronto, Toronto, Canada.
- FISHER, DR. KENNETH C., Department of Biology, University of Toronto, Toronto, Canada.
- FORBES, DR. ALEXANDER, Harvard University Medical School, Boston, Massachusetts.
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- GATES, DR. REGINALD R., Woods Hole, Massachusetts.
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# DOMINANT LETHALITY AND CORRELATED CHROMOSOME EFFECTS IN HABROBRACON EGGS X-RAYED IN DIPLOTENE AND IN LATE METAPHASE I<sup>1</sup>

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## INTRODUCTION

If oviposition is prevented in well-fed females of the parasitic wasp *Habrobracon* by withholding them from their host they continue to produce mature eggs until the egg sacs are filled. These stored eggs may number as many as twenty per female and are in late metaphase of the first meiotic division (metaphase I). Their retention in this stage for four days has no effect on their hatchability which is 96 per cent in the wild type stock used for the experiments herein described.

When unmated females with stored eggs are x-rayed and allowed to oviposit at 30° C. all eggs laid during the first six hours after treatment will have been irradiated in late metaphase I. Eggs laid during the seventh and eighth hours after treatment consist of a variable mixture treated in metaphase I and in late diplotene (including all eggs in diakinesis) and are, therefore, of no use in the present study. Eggs laid during the ninth to twelfth hours after treatment will have been post-synaptic with their diffuse chromosomes in a relatively quiescent condition when irradiated. These are designated as late and early diplotene.

An advantage in the use of these eggs for the detection of injuries lies in the fact that they develop parthenogenetically if unfertilized and so indicate directly the effects of treatment on a haploid set of chromosomes. Disadvantages are the large number ( $n = 10$ ) and small size (less than  $1\mu$  in diameter) of their chromosomes. The details of oogenesis appear to be orthodox and so the results should be universally applicable to forms with comparable type of meiosis. Failure to hatch and cytological changes in stages immediately following treatment have been the criteria of injury. Preliminary results were first published in 1938 (Whiting, 1938). Details of technique and hatchability effects, as well as extensive bibliography, are given elsewhere (Whiting, 1945); cytological effects and their correlation with mortality and dose are presented here in detail.

## DOSE-HATCHABILITY RELATIONSHIPS

Hatchability effects may be summarized briefly. No correction for control hatchability is made since it is so close to 100 per cent. Eggs x-rayed in diplotene

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and allowed to develop parthenogenetically give a dose-hatchability curve which appears to be linear at low doses and to become "mixed" at high doses; they have 50 per cent mortality at 12,000 *r* and 100 per cent at about 45,000 *r*; they showed no significant change in hatchability in preliminary and inadequate tests of time-intensity differences. Those treated in early diplotene (laid during the eleventh and twelfth hours after treatment) show no change in hatchability at any dose with fractionated treatment, those treated in late diplotene (laid during the ninth and tenth hours after treatment) show a significant increase at high doses with fractionated treatment. The dose-hatchability curve for combined diplotene owes much of its mixed character at high doses to late diplotene, early diplotene response being more nearly linear.

Eggs x-rayed in late metaphase I and allowed to develop parthenogenetically show a linear decline in hatchability with increasing dose and have 50 per cent mortality at 375 *r*, 100 per cent at about 1,400 *r*; they show no change in dose-hatchability relationships with aging between treatment and oviposition, time-intensity differences or fractionation of dose.

When *Habrobracon* females are mated, about two-thirds of the eggs are fertilized. If treated females are mated to untreated males, the survival of any appreciable number of eggs through the aid of normal spermatozoa would increase percentage of hatchability thereby indicating the presence of recessive lethal effects by comparison with hatchability of eggs from treated unmated females. Table I

TABLE I  
*Hatchability percentages for eggs of treated females, unmated and mated to untreated males*

| Stage treated | Dose in <i>r</i> units | Unmated females |                         | Mated females  |                         |
|---------------|------------------------|-----------------|-------------------------|----------------|-------------------------|
|               |                        | Number of eggs  | Hatchability percentage | Number of eggs | Hatchability percentage |
| Metaphase I   | 560                    | 319             | 39.8±2.7                | 318            | 40.5±2.7                |
| Prophase I    | 5,600                  | 137             | 71.5±2.6                | 126            | 70.6±4.0                |
|               | 22,400                 | 100             | 19.0±3.9                | 182            | 19.2±2.9                |
| Controls      | 0                      | 127             | 98.4±1.1                | 363            | 98.6±0.6                |

demonstrates that most, if not all, of the lethal effects induced in these stages by x-rays are dominant, at least in respect to hatchability. This is rather surprising at first glance but in treated metaphase I, as pointed out below, chromosomal deletions appear to be relatively large and in either stage, it is possible that deletions small enough to act as recessives in fertilized eggs may not kill the individual until after hatching in unfertilized eggs. Lethals which are recessive in diploids may be due to such minute losses as to exert their effects only after hatching in haploids. Perhaps viable deficiency heterozygotes are so rare that hatchability of irradiated eggs is not perceptibly altered by fertilization with untreated spermatozoa. In any case, conditions are well suited to an analysis of dominant lethal ratios induced by x-rays in identifiable stages of meiosis and, although the chromosomes present

difficulties, the eggs themselves are easily handled, fixed and stained for observation.

About 40,000 eggs were collected and observed for hatchability. Records were kept of the results from individual females in all cases so that aberrant behavior in eggs from any individual could be recognized. Such behavior was extremely rare.

From the work of Sax (1938, 1940), Fabergé (1940) and others on dose-chromosome injury curves, certain tentative conclusions were drawn concerning cytological effects before study of chromosomes was begun. For diplotene it was assumed that the great majority of chromosome breaks must undergo restitution; that broken ends of chromosomes within the same cell increase as dose increases, permitting complicated reunions (translocations, large interstitial deletions) so that lethal individual chromosome changes tend to be due increasingly to more than one ionization, especially in late diplotene; that bridges can be formed in either meiotic division or in both, due to lateral fusion of the broken ends of chromatids whenever two adjoining chromatids are broken by a single ionization. From the work of Sturtevant and Beadle (1936) and of McClintock (1941) it was thought that bridges in division I might be permanent or delayed in breakage and might offer an explanation for some, at least, of the high resistance of this stage to irradiation.

Concerning metaphase I it was assumed, because of the linear relationship of hatchability to dose, that injuries were in the form of terminal deletions or of minute interstitial deletions, in other words, injuries due to single ionizations. The high sensitivity of this stage suggested that most injuries must be permanent. It was doubted that a single ionization would break two chromatids due to the degree of separation in late metaphase I and so the occurrence of bridges in either division from this cause seemed improbable. There was also the possibility that high metaphase sensitivity might be due to "physiological" effects, stickiness, etc., which would result in fusion bridges, delay in division or death.

#### CYTOLOGICAL OBSERVATIONS

*Cytology of controls.* The cytology of the stages before metaphase I has not been studied in detail, either in control or irradiated material, because of the small size and large numbers of chromosomes and of their elongate and diffuse condition. Synapsis occurs in very young oocytes and the subsequent behavior through condensation appears to have nothing exceptional about it. Changes take place slowly and are not obvious in character until just before condensation of chromosomes (diakinesis) when tetrads move to periphery of the nucleus. Most students of hymenopteran cytology would question the conclusion that the stored oocyte is in an orthodox and identifiable stage, late metaphase I. They state that the chromatin has reverted to a resting stage or has formed an abortive spindle, a compact clump or a composite body, etc. Speicher (1936) finds that the most advanced eggs in the *Habrobracon* egg sac are in "early anaphase of the first maturation" which the author prefers to call late metaphase. Speicher's observations that distinct chromosomes are present, are in the form of tetrads (Fig. 1) and are ten in number has been repeatedly checked by the author and cannot be questioned. They show the

forms expected for tetrads and each resolves immediately into two pairs of dyads upon completing division I. The conclusion must be drawn either that *Habrobracon* differs from many other Hymenoptera in having orthodox oogenesis or that its chromosomes retain more easily their individuality when fixed.

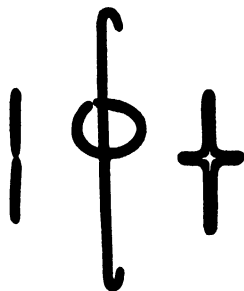


FIGURE 1. Three tetrads from one late metaphase I spindle. Untreated. Drawn from whole mount of egg with aid of a camera lucida. Semi-diagrammatic.  $\times 4,625$ .

The stages of normal oogenesis following oviposition, as described by Speicher, are briefly as follows. During the process of oviposition the maturation spindle is moved from dorsal to ventral side of the egg. It then passes into telophase I. The second division follows immediately. The four groups of chromosomes (1a, 1b, 2a, 2b) lie in a row roughly perpendicular to the egg surface. During anaphase II polar nuclei 1a and 2a remain stationary, 1b moves close to 2a, and 2b (functional nucleus) sinks deeper into the egg, a membrane forming as it moves. Nucleus 1a soon disintegrates, 1b and 2a unite and form a metaphase plate which divides and then disintegrates. Cleavage is of the usual insect type, with nuclei moving about until blastoderm formation when cell membranes first appear. The stages following oviposition are the ones which were studied after irradiation.

No evidences of displaced chromosomes or of aberrant conditions resembling those observed in irradiated eggs were found by Speicher or by the author in large numbers of control eggs studied.

*Cytology of irradiated eggs.* In experiments concerned with cytological effects, eggs from control and treated females were incubated according to standard schedules, dropped into fixative (formalin-acetic-alcohol), punctured at the posterior end to facilitate fixation, treated with the Feulgen technique and mounted whole in balsam. Control hatchability tests were made of eggs treated at the same time as those fixed. Slides were made of about 2,500 eggs.

After treatment in diplotene acentric fragments, dicentrics or both may occur in division I (Fig. 2a, b, c) or in division II or in both divisions. Bridges in division I may be permanent and can be seen bulging at the side when nucleus 1b moves towards 2a, indicating that chromatin bridges are tensile but not elastic (Fig. 2c). Acentric fragments remain visible throughout both divisions. No evidences of stickiness or of clumping of chromatin (Fig. 2a, b, c) or of retardation of meiosis are apparent for doses up to lethal (45,000 r). Of eggs treated in diplotene with 44,800 r, 1.1 per cent died at first cleavage, 30.4 per cent with a few nuclei, 54.3 per cent with many nuclei and 14.2 per cent at blastoderm.

Immediately after irradiation in late metaphase I (Fig. 1), chromosomes show no apparent change but at telophase I acentric fragments are left within the spindle and these remain visible throughout division II (Fig. 2d, e). They are often almost as large as entire chromosomes and can usually be identified as double structures. No bridges have been seen in division I in over 1,500 eggs observed. In

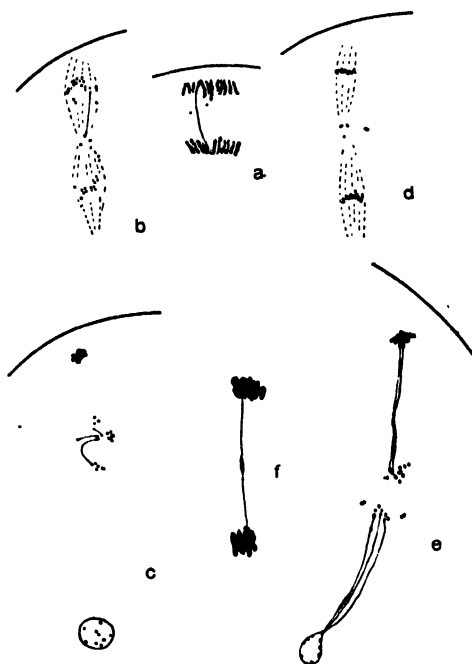


FIGURE 2. Illustrations were drawn from whole mounts of eggs with the aid of a camera lucida. *a*, *b*, and *c*, eggs were irradiated in late diplotene with 44,800 *r*; *a*, telophase I.  $\times 1,500$ ; *b*, metaphase II.  $\times 875$ ; *c*, telophase II.  $\times 1,250$ ; *d*, *e*, and *f*, eggs were irradiated in late metaphase I with 2,000 *r*; *d*, metaphase II.  $\times 875$ ; *e*, telophase II.  $\times 1,250$ ; *f*, third cleavage, telophase.  $\times 4,550$ .

division II bridges occur and after heavy treatment (2,000 *r*) several may be seen in each second division spindle (Fig. 2e). Small fragments occasionally appear in division II spindles. No evidences of stickiness or of clumping (Fig. 2d, e) or of retardation of meiosis occur in development following treatment with lethal dose of this stage which, except for absence of bridges in division I, behaves cytologically as treated diplotene. Percentages of eggs with fragments in division I and mean number of fragments per treated increase linearly with increased dose (Whiting, 1945). All eggs exposed in late metaphase I to 2,016 *r* undergo some development. 2.4 per cent die in first cleavage, 7.2 per cent with a few nuclei, 71.4 per cent with many nuclei and 19.0 per cent at blastoderm stage. In spite of their high sensitivity, some eggs treated in metaphase I developed to the fifth cleavage (expected) after 15,000 *r*, to metaphase II after 25,000 *r*, to pronucleus after 35,000 *r* and one to anaphase II after 200,000 *r*. No records were kept of rate of development at these higher doses.

The similar patterns of stage at death for both diplotene and metaphase I at their respective lethal doses indicate that, in spite of the great difference in sensitivity between the stages, cause of death is of the same nature in both. These data on time of death check what has often been noted, especially in respect to mature spermatozoa, that so-called lethal doses are not actually lethal to the treated cell itself but, instead, to its descendents. The fact that the oocyte continues to function normally and that death does not occur until it becomes an embryo, supports the argument that cytoplasmic injury is not at the basis of mortality. It is due, rather, to loss of parts of chromosomes during meiosis following irradiation and to resulting incomplete chromosome complements in every cleavage nucleus.

Bridges occur in cleavage I after treatment in either stage indicating that, if chromatids are already split when treated, there occurs lateral fusion of broken ends of half-chromatids. If they are not split when treated the split occurring in the first mitosis must have been incomplete in the broken chromatid or have resulted in lateral fusion of broken ends of daughter chromosomes. Bridges appear in subsequent cleavages. Fragments, which also occur in cleavage, could not be explained at first until it was noted that they are tapering at the ends and that they result from double breaks in a bridge which releases a thickened middle portion (Fig. 2f). Fragments were not observed after every mitosis in the same embryo although bridges, if present, appear in all cleavage figures.

#### CORRELATION OF INJURY WITH CHROMOSOME FORM WHEN TREATED

It is perhaps unwise to devote much time and space to the subject of the correlation of the nature of the injuries and the form of the chromosomes when treated in view of the small size of *Habrobracon* chromosomes and the disagreement of investigators in this field. Obviously, there is a correlation. The studies of Sax (1938, 1940), Fabergé (1940) and McClintock (1938) will be used as a basis of a brief discussion, since the results of these investigations are consistent with their theories.

Chromosome injuries fall into two classes, those caused by single ionizations and those caused by more than one. The former consist of terminal deletions and minute interstitial deletions. Two identical terminal deletions can be induced by a single ionization if two chromatids are sufficiently close together. When this happens, lateral fusion of broken ends occurs resulting in a dicentric, from parts of the two chromatids still attached to spindle fibers, and an acentric, from the released and fused ends. Single terminal deletions can be induced by single ionizations and this appears to be the rule when chromatids are widely separated. An acentric is ultimately lost and a dicentric forms a bridge when its two spindle fiber attachment points (centromeres) are pulled apart. If the bridge does not break, an entire chromosome may be missing from a daughter cell. If it does break, the resulting chromosomes are incomplete and each daughter cell will have an incomplete chromosome and, therefore, an incomplete set of genes. Such a terminally incomplete chromosome may continue to form a bridge in each subsequent division, either by failing to split completely or by a lateral fusion of the broken ends after splitting. This appears to be the general rule but McClintock (1941) has found that when such an incomplete chromosome occurs in the sporophyte tissue of maize, it forms no bridge.

An interstitial deletion caused by a single ionization in a chromosome would mean the loss of genes and would be lethal if they were numerous or of sufficient importance but it would not be cytologically apparent in subsequent divisions because of its small size.

Injuries which must be due to more than one ionization since they involve breaks in chromosomes too far apart to be caused by a single ionization are large inversions, large interstitial deletions and translocations. Inversions would not be apparent, either cytologically or in effect on viability of the embryo receiving them in the present study since they would be induced after synapsis and crossing over and the inversion of a block of genes would probably have no lethal effect. Large interstitial deletions would have a lethal effect but could not be identified in material used in these experiments. Translocations might be lethal and would be visible as bridges should centric parts of non-homologues become attached to each other. Such bridges cannot be distinguished cytologically from those resulting from double terminal deletions in this material.

The nature of the hatchability curves suggests that most injuries in early diplotene and late metaphase I at all doses and in late diplotene at low doses are caused by single ionizations, that many injuries in late diplotene at high doses are caused by more than one ionization. Since there is no reason to suppose that the nature of original breaks would be changed by higher doses it is presumed that the number of single breaks per cell increases with high dose and allows greater opportunity for new combinations because of increased number of broken ends available at any one time. This would take for granted the breaking of single chromatids per ionization for if two were broken the lateral fusion of broken ends would prevent translocations, fusion with more distant chromosomes. The reduction in injury by fractionation of dose is explained on the grounds that, with repeated smaller doses, fewer free ends are available at any given time for new combinations and the intervals between treatments afford an opportunity for restitution or changes in broken ends to occur so that they are no longer capable of joining with other broken ends formed by later treatments.

Three conditions seem to be of importance, then, in determining response of the chromosomes here studied to irradiation. These are (1) relation of tetrads to each other in the nucleus, (2) degree of separation of adjoining chromatids within a tetrad and (3) nature and degree of tension on chromosomes. Each of the three stages will be discussed briefly from these points of view.

In early diplotene the tetrads are evenly distributed within the nucleus, sister chromatids are in contact, homologues separated except at chiasmata, and neither traction of the spindle fibers nor terminalization has begun. Most breaks will be temporary because of lack of tension and relaxed state of the chromosomes. Translocations should be possible but the majority of breaks will involve both sister chromatids with the production of acentrics and dicentrics. Permanent double breaks can occur either between centromeres and proximal chiasmata (with production of bridge in division II) or distal to chiasmata (producing bridge in division I if distal to "odd" chiasmata, in division II if distal to "even") since the slight tension which exists is equally exerted everywhere along the length of the chromosome.

In late diplotene the tetrads move peripherally but are still widely separated, terminalization (movement of chiasmata towards ends of tetrads) has begun, as

well as movement of centromeres away from each other, and chromatids are not so closely associated, especially toward ends of chromosomes. Single and double breaks will occur (the latter nearer the centromere) and more of them will be permanent because of new tensions. Bridges should be less frequent in division I than in the case of early diplotene but this has not been checked. This stage will be somewhat more sensitive and will exert its lethal effects through translocations and large interstitial deletions as well as through double terminal deletions.

In late metaphase I, the tetrads are isolated from each other and stable in position on the spindle so that interchanges between them would not be expected. Centromeres are pulled far from each other and chiasmata resist further terminalization (Fig. 1) so that tension exerted between centromeres and proximal chiasmata is very great, tension exerted distal to chiasmata not so great. Ionizations will cause double breaks near centromeres where sister chromatids are closely approximated and these will all be permanent because of the extreme tension. They will result in large double fragments (acentrics) in division I; bridges (dicentrics) in division II. Breaks induced towards ends of chromosomes, and especially distal to chiasmata, will be less likely to be permanent and more likely to be single. There will be few or no bridges in division I and single fragments will appear in division I or division II (McClintock, 1938).

Any injury to a tetrad which results in a single bridge in division II reduces the chance of hatching of the egg by fifty per cent; in division I the effect is the same if the bridge breaks promptly. If it is delayed in breaking or does not break the hatchability of the egg is not affected, since an incomplete chromatid is thereby restrained from entering the ootid nucleus. A single terminal deletion reduces the chance of hatching by twenty-five per cent.

With ten tetrads of the diverse forms found in *Habrobracon*, combinations of changes induced by single ionizations can become very complex. If added to these are the complication of translocation and of large interstitial deletions (characteristic especially, perhaps, of late diplotene) the great resistance of diplotene is to be wondered at. The author (Whiting, 1945) has reviewed the data here reported in the light of the numerous theories devised to explain differential sensitivity of chromosomes to x-rays and has found that the only one which applies is that put forth by Goodspeed in 1929. He suggested tension as the important factor. It seems highly probable that numerous breaks do occur in the evenly distributed, diffuse, slowly moving chromosomes of diplotene but that the majority of them is temporary. Healing or restitution must take place quickly for there is always some movement and these chromosomes ultimately go through the same stresses as those treated in late metaphase I and, in addition, those attendant upon condensation and complete terminalization. Their response to fractionation also argues for relatively rapid restitution.

The development of individuals, normal in appearance and in reproductive activity and with normal descendents, after treatment in diplotene with 35,000 *r*, illustrates graphically the resistance of this stage to permanent injury by ionizations.

Sax (1942) summarizes the information available on "physiological" effects of x-rays, one of which is stickiness of chromatin. It has been found that condensed chromosomes are most sensitive in respect to stickiness, that such effects are temporary, delay subsequent division, have a threshold dose, are lethal only after

very high doses and result in "fusion" bridges if the cell divides before recovery. The stage in the present study most likely to show the effects of stickiness in the form of fusion bridges is division I after treatment of metaphase I. This is the only division which shows bridges of no kind even after doses much higher than lethal. A delay of twenty-four hours between treatment and resumption of meiosis does not increase hatchability, meiosis is not appreciably delayed after irradiation, there is no threshold effect (down to 50  $r$ ). It should be emphasized again that, wide apart as are the lethal doses for diplotene and metaphase I, at their respective lethal doses, the pattern of stages at death is the same, the same percentage dies at first cleavage, at blastoderm, etc.; in other words, 45,000  $r$  has no more drastic effect on development of treated diplotene than 1,400  $r$  on metaphase I. All evidence indicates that cause of death is of the same order for both stages and that "physiological" effects are of every minor importance, and not appreciably different in the two stages.

Sturtevant and Beadle (1936) failed to recover an expected genetic type of chromosome aberration correlated with a dicentric in division I. They suggested that in a form like *Drosophila* where the four meiotic nuclei lie in a row and where a terminal one alone functions, a bridge in division I might fail to break, or might be delayed in breaking, thereby tying together injured chromatids and allowing uninjured ones to pass to the terminal nuclei. McClintock (1941) also offers as explanation for the failure to obtain expected genetic results correlated with bridges in division I of maize, the selective effect of these bridges on broken chromatids. Terminal nuclei (one of which becomes the functional megaspore) tend to receive the uninjured chromatids. Figure 3c demonstrates that bridges in division I in *Habrobracon* eggs do not break, at least in some cases.

In divisions following treatment of diplotene to which this selection of injured chromatids for elimination would apply, the chances of having bridges in the second division are as frequent as in the first or more so and selection through permanence of bridges would apply, therefore, only in the simplest kind of injury and that to but one or very few tetrads since any number of breaks would be certain to produce some bridges in division. This selection, although it undoubtedly occurs, cannot explain more than a small amount of resistance of diplotene. It would be expected to apply especially with low doses when but a single break occurs in a single tetrad.

The wide difference in size of lethal doses (45,000  $r$ –1,400  $r$ ) of such closely related stages of the same cell, the unreduced *Habrobracon* egg, confirms the truth of the conclusion made long ago (1906) by Krause and Ziegler in an extensive and critical study of tissue injury by x-rays, that it is less the kind of cell than its stage at the time of treatment which determines sensitivity.

The facts and theories just presented are of interest in connection with a discussion of dominant lethals by Pontecorvo (1942). He explains dominant lethal effects in *Drosophila* spermatozoa by assuming that single chromosome breaks are produced by radiations at a rate proportional to radiation dose and that these neither undergo restitution nor participate with other breaks in the same nucleus in rearrangements. "Chromosomes with broken ends give rise to a cycle of breakage-fusion-bridge phenomena in development." He also writes, "It is therefore an open question whether sister unions are so frequent as to cause a considerable portion of dominant lethality. Should this be the case, the trend of the curve of dominant lethality could be explained. Most dominant lethality would be de-

terminated by single-break sister unions at low dosages and as the dosage increased lethal changes of the other two types (translocations and deletions) would come to play an increasing part." Translocations and deletions would not be produced actually until syngamy since breaks appear to remain open in the sperm chromosomes until that time.

Broken chromosome ends do undergo restitution or participate with other broken ends in the egg very soon after treatment but the final contribution to the zygote may be the same as that made by the irradiated sperm, viz., a chromosome with a broken end which will give rise to the breakage-fusion-bridge cycle in the first cleavage as well as in subsequent ones. Most dominant lethality in the present study is, without much doubt, caused by single ionizations and only in late diplotene at high doses does treatment appear to cause a high percentage of death from the cooperation of two or more ionizations.

It is of interest in this connection to note that the dose-injury curve for dominant lethality in the spermatozoa of *Drosophila* (Sonnenblick, 1940; Demerec and Fano, 1944) and for *Habrobracon* (Heidenthal, 1945) is of the same nature as that for late diplotene.

#### CONCLUSION

1. (Tentative) The majority of dominant lethals induced in late diplotene by low doses (to 11,000 *r*) and in early diplotene and in late metaphase I by all doses through lethal, in *Habrobracon* eggs, is caused by single ionizations which break adjoining chromatids. Lateral fusion of broken ends results, followed by continued breakage-fusion-bridge phenomena in cleavage. With doses above 11,000 *r* in late diplotene an increasing number of lethal changes arises from two or more ionizations (translocations, large interstitial deletions). 2. Lethal doses are not lethal to the treated cell (oocyte) itself but to its descendents (embryo). Fragmentation of chromosomes is not lethal, loss of fragments is. 3. The nature and degree of chromosome injury can be correlated with the form of the chromosome and with forces acting upon it during and immediately following treatment. 4. The kind of cell is less important than its stage in determining sensitivity to x-rays. 5. Tension is the main factor in determining permanence of breaks caused by ionizations, chromosome form and movement in determining the nature of the new combinations of broken ends. 6. The chromosome phenomena here dealt with are common ones in the majority of animals and plants and it is predicted that, when metaphase and anaphase are sufficiently studied in other forms, they will be found to be the stages most sensitive to x-rays. This has proved to be the case for *Sciara* (Metz and Bozeman, 1940; Reynolds, 1941) and for *Trillium* (Sparrow, 1944).

#### SUMMARY

Unlaid *Habrobracon* eggs x-rayed in diplotene (lethal dose about 45,000 *r*) and allowed to develop parthenogenetically, show fragments, bridges or both in division I; either or both in division II. Bridges in division I may be permanent.

Unlaid eggs x-rayed in late metaphase I (lethal dose about 1,400 *r*) show fragments but no bridges in division I; bridges, fragments or both in division II.

An explanation of difference in cytological effects of x-rays on these stages and of the differences between them in sensitivity and in nature of survival curves is attempted through comparison with studies on forms with larger chromosomes.

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# STRATIFICATION AND BREAKING OF THE ARBACIA PUNCTULATA EGG WHEN CENTRIFUGED IN SINGLE SALT SOLUTIONS

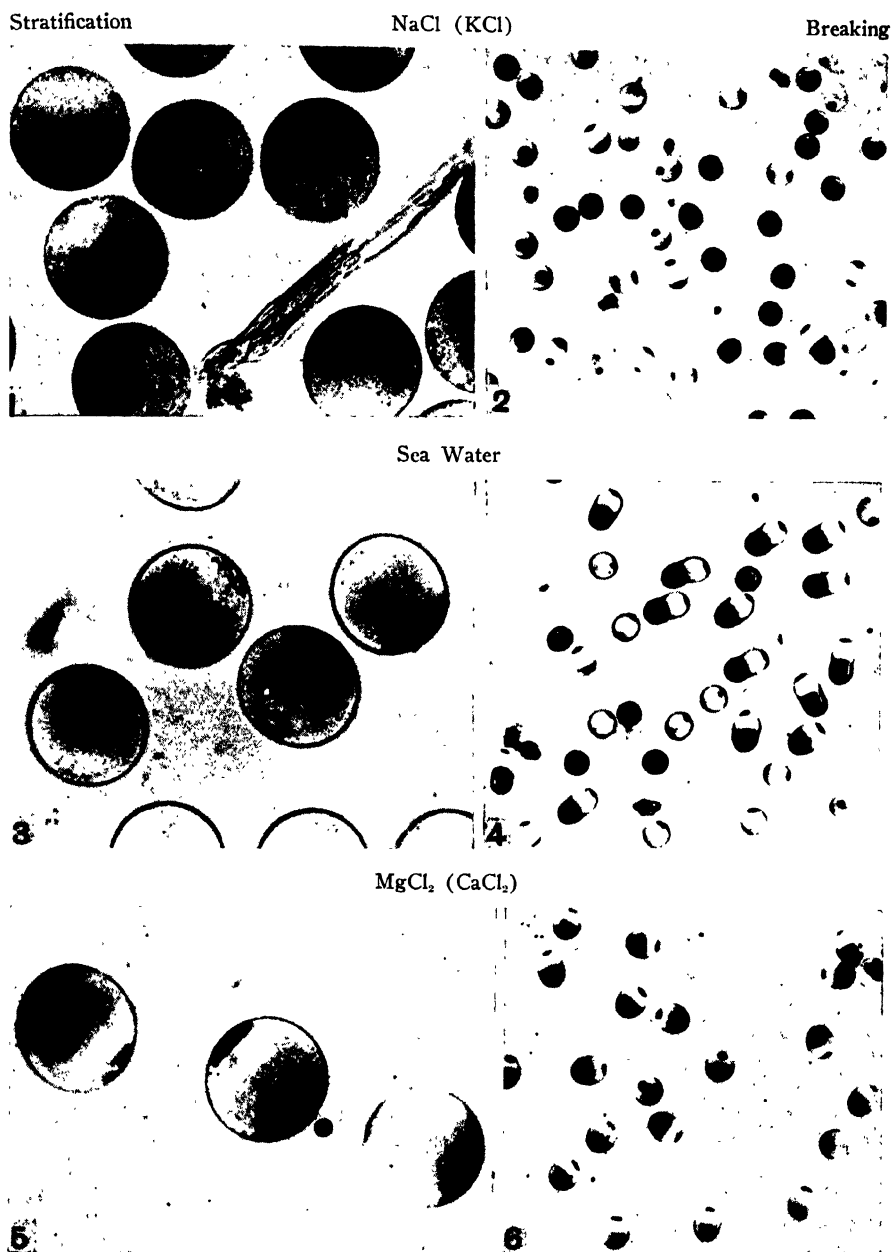
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A study has been made of the comparative rate of stratification and breaking of the *Arbacia* egg in single salt solutions, when subjected to centrifugal force. It might be expected that when more rapid stratification occurs, the eggs would break apart more readily. This was, however, found not to be the case when the eggs were centrifuged in hypo- and hypertonic sea water, but this is probably due to the change in volume of the eggs (E. B. Harvey, 1943). With the increased surface area of the eggs in hypotonic sea water the tension at the surface is increased (Cole, 1932) and the eggs are more difficult to break apart. In the present experiments with pure salt solutions the surface area remained constant.

The solutions used in the following experiments were those routinely used at Woods Hole as isotonic with the sea water there, and found by me to be isosmotic on measuring the eggs after immersion, namely: 0.52 m NaCl, 0.53 m KCl, 0.34 m  $\text{CaCl}_2$ , and 0.37 m  $\text{MgCl}_2$ . The pH of the solutions was found to be respectively, 5.54, 5.44, 5.53, and 6.31. It was determined, however, that the pH in itself, at least of sea water, has no effect on the stratification and rate of breaking. Sea water was made up of pH ranging from 5 to 9 by adding HCl or NaOH; eggs kept in these solutions and centrifuged in them at the same time as those in normal sea water showed no difference in stratification or breaking. This was found also by Barth (1929) for stratification in sea water, though he did find an effect in NaCl. However Heilbrunn (1928, 1943) finds that Na definitely increases viscosity. The eggs were not injured by the pure salt solutions as they could be fertilized on removal to sea water after 40 minutes in the solutions and produced normal plutei. However, the eggs cannot be fertilized while in the solutions; the sperm are immotile in all except NaCl, and here no fertilization membrane was seen.

*Arbacia punctulata* eggs were placed in 50 cc. of the isosmotic salt solution for 20 minutes and this was replaced by a fresh salt solution for another 20 minutes. Three tubes of experimental eggs (in different salt solutions) and one tube of control eggs (in sea water) were centrifuged at the same time; isosmotic sugar solution was placed in the bottom of each tube to keep the eggs suspended. Care must be taken that exactly the same amount of sugar solution is used in each tube and exactly the same amount of egg suspension placed on top, so that the eggs in each tube are thrown to the same level and are subjected to exactly the same amount of centrifugal force. For stratification the force used was about  $3,000 \times g$  for two minutes, and for breaking  $10,000 \times g$  for four minutes. Each experiment was repeated many times. A single batch of eggs was always used in each experiment.



## EXPLANATION OF PLATE

Stratification of *Arbacia punctulata* eggs centrifuged at  $3,000 \times g$  for two minutes in (1) NaCl, (3) sea water, (5) MgCl<sub>2</sub>. Breaking apart of eggs at  $10,000 \times g$  for four minutes in (2) NaCl, (4) sea water, (6) MgCl<sub>2</sub>. KCl acts much like NaCl and CaCl<sub>2</sub> much like MgCl<sub>2</sub>.

The experiments were carried out at approximately 23° C., so that the temperature effect observed by Costello (1934, 1938) was not involved.

It was found that in the monovalent salts, NaCl and KCl, the rate of stratification is less than in sea water, and in the bivalent salts, CaCl<sub>2</sub> and MgCl<sub>2</sub>, the rate of stratification is greater than in sea water (Photographs 1, 3, 5). The viscosity, then, is increased in NaCl and KCl and decreased in CaCl<sub>2</sub> and MgCl<sub>2</sub>. In the effect on the rate of stratification the series runs, from most to least: Ca > Mg > S.W. > Na > K. This is similar to the series given by Heilbrunn (1923, 1928) in a slightly different experiment with *Arbacia* eggs, except that Na and K are reversed. This is possibly due to a difference in the tonicity of the solutions used. His series for *Stentor* is the same as my series for *Arbacia*.

In ease of breaking with centrifugal force, the series runs in the reverse order. Eggs in KCl, where the stratification is least in a given time, break most readily, and those in CaCl<sub>2</sub>, where the stratification is greatest, break least readily. Eggs in the monovalent salts, NaCl and KCl, break more readily than those in sea water while the eggs in the bivalent salts, MgCl<sub>2</sub> and CaCl<sub>2</sub>, break less readily than those in sea water (Photographs 2, 4, 6). In ease of breaking, the series runs, from greatest to least: K > Na > S.W. > Mg > Ca. The ease of breaking has been judged by the percentage of eggs broken in a given time with a constant force, rather than by the time for a definite percentage to break, since the experiment can be carried out more accurately when experimental and control eggs are centrifuged at the same time. An average experiment gives the following figures for percentage of eggs broken when centrifuged for four minutes at 10,000 × g.

|     |      |           |                   |                   |
|-----|------|-----------|-------------------|-------------------|
| KCl | NaCl | Sea water | MgCl <sub>2</sub> | CaCl <sub>2</sub> |
| 99% | 90%  | 50%       | 20%               | none              |

There was no measurable difference in the relative size of the two "halves" in any of the pure salt solutions; the white and red "halves" were the same size as those obtained when eggs were kept and centrifuged in sea water.

There is considerable variation in ease of breaking in different lots of eggs with the same centrifugal force, and even the same batch varies slightly after being kept in sea water for several hours. In one experiment 98 per cent were broken in sea water, and 40 per cent in CaCl<sub>2</sub>; in another experiment, 50 per cent were broken in NaCl and 20 per cent in sea water. In every experiment, however, the eggs in the solutions broke in the order named. It was thought that possibly the jelly surrounding the eggs might be influenced by the salt solutions and be responsible for the difference in ease of breaking. Jelly was found to be present on the eggs in all the solutions. Eggs from which the jelly was removed by addition of 0.2 cc. N/10 HCl to 50 cc. sea water, and then well washed in sea water broke in the solutions in the same order as those with jelly.

Since the experimental results are contrary to the expectation that the interior viscosity is the controlling factor in breaking of the eggs, we are led to the conclusion that the salts affect the "tension at the surface." Despite the increased interior viscosity in pure NaCl and KCl, the surface forces resisting the pulling apart of the eggs are actually decreased. In CaCl<sub>2</sub> and MgCl<sub>2</sub> they are increased though the interior viscosity is decreased. Heilbrunn (1923, 1943) has pointed out that in *Amoeba*, and apparently also in *Arbacia* eggs, the cortical protoplasm

reacts differently from the interior protoplasm, and Brown (1934) has found a difference in cortical and interior protoplasm in response to hydrostatic pressure on fertilized *Arbacia* eggs.

An effect on the surface forces without any effect on the interior viscosity is given by eggs in Ca-free sea water. Unfertilized eggs kept and centrifuged in Ca-free sea water stratify at the same rate as those in sea water, as shown in previous experiments with a double image centrifuge microscope (E. B. Harvey, 1933). They break apart more readily in Ca-free sea water than in normal sea water—at about the same rate as those in NaCl alone. The fertilized eggs also break more readily in Ca-free sea water than in normal sea water, as shown previously. The absence of calcium therefore tends to decrease the surface forces and the presence of calcium alone tends to increase them. That calcium has an effect on the surface layers of eggs is well known, and has been especially emphasized by Heilbrunn (1928, 1943). A very good example is given by the classic experiments of Herbst (1900) in separating blastomeres due to the dissolution of the ectoplasmic (hyaline plasma) layer in Ca-free sea water.

#### SUMMARY

When unfertilized *Arbacia punctulata* eggs are centrifuged in isosmotic single salt solutions, they stratify with decreasing readiness (indicating increasing viscosity) in the following order:  $\text{CaCl}_2 > \text{MgCl}_2 > \text{S.W.} > \text{NaCl} > \text{KCl}$ . They break into "halves" with decreasing ease in the reverse order, those in  $\text{CaCl}_2$  which stratify best, break least readily. In the bivalent salts they stratify better and break less readily than in sea water, and in the monovalent salts they stratify less and break more readily than in sea water. The ease of breaking must be determined by an effect of the salts on the surface layers rather than by their effect on the interior viscosity.

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# THE EFFECT OF CYANIDE ON RESPIRATION IN *PARAMECIUM* CAUDATUM AND *PARAMECIUM* AURELIA<sup>1</sup>

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In some ciliates the presence of a cytochrome-oxidase system has been established. Pitts (1932) claimed that *Colpidium campylum* showed an initial sensitivity to HCN but that the oxygen consumption soon increased until it even surpassed normal consumption. Lwoff (1934) also found an initial inhibition followed by an acceleration in respiration in another ciliate, *Glaucoma pyriformis*, when it was exposed to KCN. Hall (1941) definitely established that HCN inhibits respiration in *Colpidium campylum* and Baker and Baumberger (1941) found that HCN inhibits respiration in *Tetrahymena geleii*.

*Paramecium* is usually cited as one of the several exceptions to the rule that most animal cells are sensitive to HCN. In fact, ciliates as a group have been regarded by some investigators as being insensitive to cyanide, although very few species have been tested. Lund (1918), Shoup and Boykin (1931), and Gerard and Hyman (1931) found that *Paramecium caudatum* was resistant to cyanide. However, Child (1941) refers to unpublished data obtained by Hyman, in which she found a considerable decrease in O<sub>2</sub> consumption of *Paramecium* in KCN. Dr. Hyman<sup>2</sup> has also informed the author by personal communication that she

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<sup>2</sup> Dr. Libbie H. Hyman has granted me the privilege of using the following communication which she sent to me at my request: "Some years ago, being skeptical of Lund's failure to find any cyanide-sensitive respiration in *Paramecium*, I spent a great deal of time and effort in testing the action of cyanide on the oxygen consumption of *Paramecium*, using Winkler's method. I met with so many difficulties that I never published the results; chief among them were the impossibility of measuring equal suspensions of *Paramecium* from a volumetric pipette because the animals adhere to the glass, and the toxicity to *Paramecium* of all waters except the culture water, which in itself has high oxygen consuming powers. However, my results indicated that starved *Paramecium* have no cyanide-sensitive respiration, in agreement with the finding of Lund, but non-starved ones have about 35 per cent such respiration. After giving up the work as impractical by my methods, I sought the help of Dr. Gerard. Dr. Gerard kindly consented to test the matter on his manometers but failed to find any depressing action of cyanide on non-starved *Paramecium*. As I played no role in this work except that I furnished the *Paramecium*, I feel that Dr. Gerard was over-generous in making me co-author. I was not satisfied with these results, first, because successive manometric readings were highly variable, and second, because the buffer solution used was toxic to *Paramecium*, depressing oxygen consumption by about 50 per cent in itself.

"As a cyanide sensitivity of the extra oxygen consumption caused by feeding was indicated in my experiments, it became interesting to know the nature of this extra respiration. I therefore attempted to compare the effects on oxygen consumption of the ingestion by *Paramecium* of particles without food value (carbon suspension) and of particles with food value (yeast). Here, again, I met with insuperable difficulties. I could never get any sample of yeast, no

found an inhibition of  $O_2$  consumption in *P. caudatum* when it was exposed to HCN.

Sato and Tamiya (1937) claimed that they found cytochrome a and c in *Paramecium*. If this is true, then it is difficult to understand the insensitivity of the respiratory mechanism of this species to HCN. Because of these observations and of the unpublished results of Hyman, and since studies have not been made on the sensitivity of *Paramecium* to cyanide when proper KOH-KCN mixtures are used as absorption media (Krebs, 1935), the following investigation was carried out.

#### MATERIAL AND METHODS

Two species were used in this work, *Paramecium caudatum* and *Paramecium aurelia*. The culture solution used was highly buffered and was the same as was used later in the flasks of the Barcroft-Warburg apparatus for testing. The solution consisted of  $K_2HPO_4 \cdot H_2O$  — 80 mg.,  $KH_2PO_4$  — 80 mg.,  $CaCl_2$  — 104 mg.,  $Mg_3PO_4$  — 2 mg., and redistilled water to make one liter.

In making up the stock culture, 15 gms. of timothy hay were boiled in 500 ml. of this solution for one-half hour, after which the solution was made up to its original volume by the addition of distilled water. This "broth" was then diluted further by the addition of the above buffered solution to make 4000 ml. The hydrogen ion concentration was held at  $pH\ 7.0 \pm 0.2$ .

This culture solution, along with approximately 3 gms. of sterile hay, was put into chemical bottles with 500 ml. capacity and moderately narrow necks (3–4 cm. in diameter). About 4000 paramecia were added to each container. Within 5 days they became extremely numerous, especially in the neck region of the bottle whence they could be removed easily in large numbers.

The Barcroft-Warburg apparatus was used for ascertaining rate of oxygen consumption. The shaking mechanism was adjusted to operate at 110 complete cycles per minute. Because of the possibility of  $NH_3$  production (Specht, 1934), a 0.3 ml. portion of 0.3 N HCl was added to the side arm (onset) of each manometer flask.

During the course of these investigations, various test solutions were made up containing different concentrations of KCN as follows: 0,  $10^{-5}$ ,  $10^{-4}$ , and  $10^{-3}$  M. Corresponding KOH-KCN absorption solutions were made up for each concentration of test solution according to Krebs (1935), and 0.4 ml. of the proper mixture (Pace and Belda, 1944) was added to the inner well (inset) of each flask containing organisms in KCN. To the inset of each of the flasks in which the test solution contained no KCN, a 0.4 ml. portion of M KOH was added.

A typical test was made in the following manner: Paramecia were drawn off from the top of the bottles in which they were cultured and placed in 15 ml. centrifuge tubes in which they were washed several times in fresh solution by careful centrifugation. The only time the organisms were subjected to centrifugation was

matter how many times boiled and centrifuged, that did not have high oxygen consuming powers, and all carbon suspensions also remove oxygen from the medium. However, there were indications that ingestion of a non-nutritive substance can cause as great an increase in oxygen consumption as does ingestion of food. This suggests that the extra respiration of feeding does not result from an oxidation of the food material."

during the washing process and this was carried out with great care by means of a hand centrifuge. An attempt was made to have between 2000 and 3000 *P. aurelia* or 1000 and 2000 *P. caudatum* in each 5 ml. sample. A count was always made of the organisms in each flask at the end of an experiment.

In all the tests reported here, those organisms designated "young" paramecia were taken from 5-7 day-old cultures; those designated "old" paramecia, from 15-20 day-old cultures; those designated "starved" paramecia were "old" organisms that had been placed in inorganic buffer solution without food material for 2 or 3 days. The "young" paramecia had much more food material present in the form of food vacuoles than the "old" paramecia.

## RESULTS

### *Effect of cyanide on respiration in Paramecium aurelia*

*Paramecium aurelia* was the first species studied. It is a much smaller form than *P. caudatum*, but its rate of respiration per unit volume is similar to the latter (Pace and Kimura, 1944).

A number of tests were made at various KCN concentrations. Organisms that were taken from cultures 15-17 days after they had been started (i.e., "old" paramecia) were used in most of the tests. They were washed by centrifugation in the solution given above, and then divided into two portions. KCN was added to one of these portions in the concentrations designated in the table. Several tests were also carried out on starved paramecia and young paramecia. The results are presented in Table I.

*P. aurelia* was found to be sensitive to KCN in all the tests made, except where starved individuals were used. The normal average oxygen consumption for organisms taken from the 15 or 17 day-old cultures was 6.31 mm<sup>3</sup> per hour per mm<sup>3</sup> of cell substance at 25° C. This compares favorably with the results of Pace and Kimura (1944) who found that *P. aurelia* consumed oxygen at the rate of 6.16 mm<sup>3</sup> per hour per mm<sup>3</sup> of cell substance at 25° C.

The presence of food material may have something to do with the fact that in all the tests made, the younger paramecia showed a much greater sensitivity to cyanide than the older. In fact, starved specimens were insensitive to cyanide. When exposed to KCN at a concentration of 10<sup>-4</sup> M, respiration in the young organisms was inhibited on the average by about 40 per cent. The respiration of old organisms showed an average inhibition of 22 per cent to the same concentration of KCN. At KCN concentrations of 10<sup>-3</sup> M, inhibition of respiration was greater than with the lower concentration, but the results were similar insofar as young and old organisms are concerned. In young paramecia, the average O<sub>2</sub> consumption (1318 mm<sup>3</sup> O<sub>2</sub> per hour per million) in the buffered solution without KCN was about twice that in old organisms. An average O<sub>2</sub> consumption of 640 mm<sup>3</sup> was found for the young paramecia when they were exposed to 10<sup>-3</sup> M KCN. Thus the cyanide at this concentration results in a 50 per cent inhibition in respiration in *P. aurelia*.

### *Effect of KCN on respiration in Paramecium caudatum*

*Paramecium caudatum* has been studied to a much greater extent than *P. aurelia* and, as brought out previously, all the work (except for unpublished early

TABLE I

The effect of KCN on respiration in *Paramecium aurelia*. \*, starved specimens; 5-7 day cultures, young specimens; all others, old specimens. Temperature, 25° C.; pH, 7.0 ± 0.2. Average volume of one million paramecia, 121.4 mm.<sup>3</sup> (this does not include the volume of starved specimens). Each figure in columns 4 and 5 represents the average for 3 tests.

| Molar concentration of KCN | Age of culture in days | Duration of test in hours | Average O <sub>2</sub> consumption in mm. <sup>3</sup> per hour per million | Average O <sub>2</sub> consumption in mm. <sup>3</sup> per hour per mm. <sup>3</sup> of cell substance | Per cent inhibition |
|----------------------------|------------------------|---------------------------|---|--|---------------------|
| 0<br>10 <sup>-4</sup>      | 17*                    | 4                         | 462<br>484  |  | None                |
| 0<br>10 <sup>-4</sup>      | 16                     | 3                         | 746<br>598  | 6.14<br>4.92   | 19.9                |
| 0<br>10 <sup>-4</sup>      | 16                     | 3                         | 680<br>485  | 5.60<br>3.99   | 29                  |
| 0<br>10 <sup>-4</sup>      | 15                     | 5                         | 709<br>453  | 5.84<br>3.73   | 36.1                |
| 0<br>10 <sup>-4</sup>      | 15                     | 3                         | 808<br>665  | 6.65<br>5.47   | 18                  |
| 0<br>10 <sup>-4</sup>      | 15                     | 3                         | 841<br>747  | 6.92<br>6.15   | 12                  |
| 0<br>10 <sup>-4</sup>      | 7                      | 3                         | 906<br>657  | 7.46<br>5.42   | 28.5                |
| 0<br>10 <sup>-4</sup>      | 5                      | 5                         | 1360<br>788   | 11.20<br>6.49  | 42                  |
| 0<br>10 <sup>-3</sup>      | 16*                    | 3                         | 520<br>511  |  | None                |
| 0<br>10 <sup>-3</sup>      | 15                     | 3                         | 818<br>557  | 6.73<br>4.58   | 32                  |
| 0<br>10 <sup>-3</sup>      | 5                      | 3                         | 1516<br>605   | 12.48<br>4.98  | 60                  |
| 0<br>10 <sup>-3</sup>      | 6                      | 5                         | 1120<br>677   | 9.22<br>5.57   | 40                  |

results of Dr. Libbie H. Hyman) indicates that *P. caudatum* is insensitive to cyanide. One great difference in the work reported here and previous investigations carried out on the effect of cyanide on *Paramecium* is that in these experiments suitable KCN-KOH absorption mixtures rather than pure KOH were used in the manometer flasks to prevent absorption of HCN from the test solution.

The same procedures were followed here as for *P. aurelia*. The results are presented in Table II.

As indicated by the results, much variation was found in the action of KCN on *Paramecium caudatum*. In the first few tests very great difficulty was experi-

TABLE II

The effect of KCN on oxygen consumption in *Paramecium caudatum*. \*, starved specimens; 5 day cultures, young specimens; all others, old specimens. Temperature, 25° C.; pH, 7.0 ± 0.2. Average volume of one million paramecia, 591 mm.<sup>3</sup> Each figure in columns 4 and 5 represents the average for 3 tests.

| Molar concentration of KCN | Age of culture in days | Duration of test in hours | Average O <sub>2</sub> consumption in mm. <sup>3</sup> per hour per million | Average O <sub>2</sub> consumption in mm. <sup>3</sup> per hour per mm. <sup>3</sup> of cell substance | Per cent inhibition |
|----------------------------|------------------------|---------------------------|---|--|---------------------|
| 0<br>10 <sup>-5</sup>      | 16*                    | 3                         | 1565<br>1518  |  | None                |
| 0<br>10 <sup>-5</sup>      | 16                     | 2                         | 3273<br>2734  | 5.53<br>4.62   | 15.5                |
| 0<br>10 <sup>-5</sup>      | 16                     | 6                         | 3734<br>3181  | 6.33<br>5.37   | 15                  |
| 0<br>10 <sup>-5</sup>      | 5                      | 9                         | 4420<br>2650  | 7.47<br>4.48   | 30                  |
| 0<br>10 <sup>-4</sup>      | 17                     | 3                         | 3040<br>3010  | 5.14<br>5.09   | None                |
| 0<br>10 <sup>-4</sup>      | 19                     | 5                         | 2700<br>1978  | 4.56<br>3.34   | 27                  |
| 0<br>10 <sup>-4</sup>      | 15                     | 3                         | 3787<br>2243  | 6.40<br>3.80   | 40                  |
| 0<br>10 <sup>-4</sup>      | 5                      | 4                         | 4270<br>2475  | 7.22<br>4.18   | 42                  |
| 0<br>10 <sup>-3</sup>      | 16*                    | 5                         | 1190<br>1280  |  | None                |
| 0<br>10 <sup>-3</sup>      | 15                     | 3                         | 3580<br>2072  | 6.05<br>3.50   | 42                  |
| 0<br>10 <sup>-3</sup>      | 5                      | 12                        | 4590<br>1560  | 7.76<br>2.63   | 66                  |
| 0<br>10 <sup>-3</sup>      | 15                     | 4                         | 4170<br>2380  | 7.05<br>4.02   | 43                  |

enced, chiefly because some apparently minor details in manipulation were overlooked and this may have had a very noticeable effect on the results. It was suspected from the results of the first few tests that food played an important part in the degree of sensitivity of these organisms to KCN. For this reason several tests were conducted on this species under the same type of conditions as was used for *P. aurelia*, namely: (1) young paramecia (5 day cultures), (2) old paramecia (15 to 19 day cultures) and (3) starved paramecia.

The results indicate that although there was great variation in some of them, the young specimens show a greater sensitivity to KCN. The starved specimens proved to be non-sensitive. In some tests there appeared to be an actual accelera-

tion of  $O_2$  consumption when starved *P. caudatum* was put into KCN solutions but the results may have been due to experimental error. They are not included in the table. In one test (included in table) which was made upon old organisms, there was no evidence of cyanide sensitivity; no explanation can be given for this exception.

The average inhibition of  $O_2$  consumption found in old *P. caudatum* exposed to solutions containing  $10^{-5}$  M KCN was approximately 15 per cent; in solutions containing  $10^{-4}$  M, 33 per cent; and in solutions containing  $10^{-3}$  M, 42 per cent. In young *P. caudatum* exposed to  $10^{-5}$  M KCN, respiratory inhibition was approximately 30 per cent; in solutions containing  $10^{-4}$  M KCN, 42 per cent; and in solutions containing  $10^{-3}$  M, approximately 66 per cent. Thus, inhibition of oxidative metabolism increases with increase in KCN concentration, and the degree of sensitivity to cyanide seems to depend upon the quantity of food material present. This is in agreement with the results of Hyman. Higher concentrations than  $10^{-3}$  M KCN were attempted but the results are meaningless because of such extreme variations and for this reason they have not been included in this report.

#### *Effect of dextrose on the degree of inhibition by cyanide*

Many workers have reported that one of the factors in the sensitivity of the respiratory mechanism to cyanide is the degree of carbohydrate saturation in the cell. Keilin (1932) suggests that perhaps the most important factor concerned with cellular sensitivity to cyanide is the concentration of carbohydrate. Commoner (1939) working with bakers' yeast, Emerson (1927) with *Chlorella*, and Hall (1941) with *Colpidium campylum*, all found either a greater inhibition with cyanide when dextrose was present or no inhibition without dextrose.

Since it is highly probable that a large portion of the food material of *Paramecium* is carbohydrate and since it was found that the greatest sensitivity to cyanide occurred when the greatest quantity of food was present, it was thought advisable to run respiration tests with the organisms in a dextrose solution.

Old paramecia were selected and washed in the buffered test solution containing 0.01 M dextrose. Then the solution containing the paramecia was divided

TABLE III

The effect of KCN on *Paramecium caudatum* in a 0.01M dextrose-buffer solution. All the organisms were taken from 16 to 19 day-old stock cultures. Temperature,  $25^\circ C$ .; pH,  $7.0 \pm 0.2$ . Average volume of one million paramecia, 580 mm.<sup>3</sup> Each figure represents the average for 3 tests.

| Molar concentration of KCN | Age of culture in days | Duration of test in hours | Average $O_2$ consumption in mm. <sup>3</sup> per hour per million | Average $O_2$ consumption in mm. <sup>3</sup> per hour per mm. <sup>3</sup> of cell substance | Per cent inhibition |
|----------------------------|------------------------|---------------------------|--|---|---------------------|
| 0                          | 16                     | 4                         | 4550   | 7.84  | 48                  |
| $10^{-4}$                  |                        |                           | 2360   | 4.06  |                     |
| 0                          | 16                     | 5                         | 3860   | 6.65  | 51                  |
| $10^{-4}$                  |                        |                           | 1890   | 3.25  |                     |
| 0                          | 19                     | 3                         | 4120   | 7.10  | 54                  |
| $10^{-4}$                  |                        |                           | 1895   | 3.26  |                     |

into two portions. To one portion, KCN was added to  $10^{-4}$  M; the other portion was used as control. This experiment was repeated twice and the results are presented in Table III.

The results show that the rate of respiration in *Paramecium caudatum* is increased with the addition of dextrose to the test solution. The average rate of respiration in the dextrose-buffer solution for all tests without KCN added was  $4170 \text{ mm}^3$  per hour per million organisms as compared to an average  $3470 \text{ mm}^3$  in the same type of organisms tested in the buffer solution without dextrose (Table II). They also show that there was an average inhibition of 51 per cent in  $\text{O}_2$  consumption in  $10^{-4}$  M KCN in the dextrose-buffer solution which is much greater than the average inhibition in  $10^{-4}$  KCN without dextrose. The average inhibition for two experiments in which the latter solution was used, was 33.5 per cent; in one of the experiments there was no inhibition whatever, but this has not been included in the average.

#### DISCUSSION

Many factors may have contributed to the failure of earlier investigators to find inhibition in respiration in *Paramecium* when exposed to cyanide. Considerable error must have been caused by the absorption of free HCN by the KOH used as absorption fluid. The initial inhibitory effect followed by an increase in oxygen consumption noted in the results of Pitts (1932) and Lwoff (1934) is evidently due to the fact that little attention was given to the rapid absorption of cyanide (via distillation of HCN) by the absorption fluid. Hall (1941), using suitable KOH-KCN mixtures as absorption media, proved conclusively that respiration in *Colpidium* was cyanide sensitive.

In the investigations reported here, care was taken to prevent distillation of HCN over into the absorption fluid. However, there is another factor that may or may not have been realized by these earlier workers, namely, the food content of the paramecia with which they worked. It is possible that the organisms used by them were taken from "old" cultures and hence had comparatively little food material in them. If this be true, it explains their failure to find inhibition in respiration, for, as reported above, sensitivity seems to depend, at least partly, upon the food content of *Paramecium*. This very important factor was noted some twenty years ago by Dr. Libbie Hyman (see footnote 2).

In these experiments, the organisms were taken from the culture solution, washed, and placed in fresh test solution, and then put into manometer flasks, all within 10–15 minutes. Thus in most of the tests the organisms were actually in inorganic solution without food for 3.5 hours; in some tests 4.5–5.5 hours, but rarely longer than this. During this time, very little change could be noted in food vacuole content or size. It was also noted that respiration varied very little, if at all, from the beginning to the end of a test. In other words, the decrease in food content is so slight within this short period of time that there was no noticeable change in rate of respiration.

Carbohydrate makes up a great portion of the food of *Paramecium*. One of the most important factors in the degree of sensitivity of respiration to KCN, etc. is the concentration of carbohydrate in the cell. Thus when dextrose was added, to the buffer solution in which the respiration of *Paramecium caudatum* was tested, the per cent inhibition was greater than in the buffer solution without dextrose.

## SUMMARY

1. The oxygen consumption in *Paramecium caudatum* and *Paramecium aurelia* is partially inhibited by potassium cyanide.
2. The extent of inhibition by cyanide is dependent upon the food content of the organisms as well as upon the concentration of cyanide in the solution.
3. In *P. aurelia*, starved specimens are insensitive to cyanide; old specimens are not as sensitive as young. In  $10^{-4}$  M KCN respiration in the old organisms was inhibited by approximately 22 per cent while in the young organisms it was inhibited by approximately 40 per cent.
4. In *Paramecium caudatum*, starved specimens were non-sensitive to KCN; old specimens exposed to  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$  M KCN show, respectively, a 42, 33, and 15 per cent inhibition in respiration. Young specimens, exposed to  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$  M KCN show, respectively, a 66, 42, and 30 per cent inhibition.
5. The inhibition in the rate of respiration in *P. caudatum* was greater in buffer solution plus dextrose (0.01 M) than in the same solution without dextrose.
6. The effect of cyanide on respiration in *Paramecium* depends upon the degree of saturation of the respiratory mechanism with carbohydrate.

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# THE AGGLUTINATION OF STARFISH SPERM BY FERTILIZIN<sup>1</sup>

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Agglutination of starfish sperm by specific egg water (supernatant sea water from egg suspensions) has never been clearly demonstrated. Glaser (1914) and Woodward (1918) reported a strong agglutination of *Asterias forbesii* sperm by homologous egg water, but Just (1930) was unable to confirm this work. Attempts to demonstrate agglutination of sperm by egg water in other species of starfish have failed. Thus Loeb (1914) observed no reaction in *Asterias* (probably *Pisaster*) *ochraceus*, and Tyler (1941) had a similar result with *Patiria miniata*. From this it might appear that fertilizin is not present in starfish egg water. However, Tyler found that treatment of *Patiria* sperm with egg water lowered the fertilizing power of the sperm. Tyler (1941, 1942) interpreted this as support for his view that fertilizin may exist naturally in a non-agglutinating "univalent" form. An individual molecule of such univalent fertilizin should have but one combining group capable of reacting with groups (antifertilizin) on the sperm surface. On the basis of the Marrack-Heidelberger (1938) lattice theory, univalent fertilizin should therefore combine with but not agglutinate these cells. Tyler suggests that such univalent fertilizin may be present quite generally in forms showing no agglutination of sperm by egg water. He therefore supports the belief held by Lillie (1919) and Just (1930) that fertilizin occurs universally.

In view of the concept of univalent fertilizin and the provisional status of the starfish with respect to sperm agglutination by egg water, it is of some interest that sperm of certain starfish agglutinate when mixed with homologous egg water and an "adjuvant." The first adjuvant found was lobster (*Panulirus*) serum. The agglutination reaction was discovered accidentally in the course of studies on the natural agglutinins in lobster serum (Tyler and Metz, 1944). In an attempt to separate natural agglutinins for *Patiria* eggs and sperm, the serum was treated with eggs and then titrated for sperm agglutinins. The treatment with eggs increased the sperm agglutinin titer several fold. Investigation of this unexpected result showed that sperm absorbed lobster serum (freed of natural sperm agglutinins), when mixed with *Patiria* egg water, agglutinated *Patiria* sperm. Tests on other material showed the presence of adjuvant in hen's egg white. A preliminary report (Metz, 1944) on this work has already appeared. The studies confirm Tyler's view that fertilizin is present in *Patiria* egg water. However, the experiments indicate that this fertilizin is multivalent. Data are given which suggest that normal *Patiria* sperm is "univalent" with respect to exposed antifertilizin groups, but that more of these groups are "exposed" by the adjuvant.

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## MATERIAL AND METHODS

The Pacific webbed star, *Patiria miniata*, was used as standard material. The Pacific star *Pisaster ochraceus*, the Pacific sand star *Astropecten* sp. and the Atlantic *Asterias forbesii* were used in confirmatory and specificity tests.

Egg and sperm suspensions were prepared from ripe extirpated gonads. These organs were minced in a measured volume of sea water and then filtered through bolting cloth to remove the gonadal tissue. The difference in volume of the filtrate and the sea water initially added gives the volume of "dry" (undiluted) material. Egg and sperm dilutions were reckoned from this "dry" volume. Egg water solutions were obtained by drawing off the supernatant from standing egg suspensions (25–50 per cent of dry eggs in sea water), or by heating such suspensions and filtering or centrifuging off the eggs.

Lobster (*Panulirus interruptus*) serum was obtained by drawing blood from the heart and allowing it to clot. After syneresis the serum was drawn off. Since *Panulirus* serum contains natural heteroagglutinins for sperm of various organisms (Tyler and Metz, 1944) including *Patiria*, *Pisaster* and *Astropecten*, it is impractical to use the untreated serum. By absorption with *Patiria* sperm the natural agglutinins for *Pisaster* and *Astropecten* as well as *Patiria* sperm can be removed. Such absorbed serum was used as the adjuvant for sperm of all three species. For reasons of economy both in material and time, hen's egg white was used as the adjuvant in the later experiments. This material was made isotonic by adding one volume of concentrated ( $1.73 \times$ ) sea water. It was then diluted to 20 per cent with normal sea water and filtered to remove the mucin, chalazae and other insoluble material. This diluted egg white was usually heated to 100° C. and filtered or centrifuged since this procedure increased its activity several fold. Hen's egg white does not contain natural agglutinins for *Patiria*, *Pisaster* or *Astropecten* sperm. Thus, initial absorption with starfish sperm was not necessary.

Assays of unknown egg water were made by diluting the unknown solution in twofold steps with sea water and then adding constant amounts of adjuvant-treated sperm to each dilution of unknown egg water. Adjuvant was titrated in a similar manner. However, when titrating adjuvant, constant amounts of sperm suspension were added to the dilutions of unknown adjuvant. Subsequently, constant amounts of egg water were added to each adjuvant dilution. Less satisfactory results are obtained if any other order of mixing is employed in this test. In all cases the presence or absence of agglutination was determined by microscopical examination one to several minutes after mixing. Titers were recorded as the highest dilution of unknown showing agglutination of the test sperm.

The apparatus and methods used in ultraviolet irradiation have been described in a previous article (Metz, 1942).

## ACTIVATION AND AGGLUTINATION OF STARFISH SPERM

In these studies no definite agglutination of *Patiria*, *Pisaster*, *Astropecten* or *Asterias* sperm was observed following the addition of homologous egg water. However, *Patiria* sperm suspensions frequently appeared "granular" after this treatment. These microscopic "granules" consisted of two or three sperm fixed together and represent a plus-minus agglutination reaction. Various devices such

as centrifugation were employed in an attempt to bring this reaction to a distinct agglutination, but all of them failed.

The starfish sperm in dilute (0.5 to 1.0 per cent) sea water suspension were virtually immobile. The cells did not respond to treatment with fresh sea water (lowering CO<sub>2</sub> tension) or with homologous egg water. Starfish sperm thus differ from *Arbacia* and *Nereis* sperm which become more active when diluted with sea water (Lillie, 1913; Just, 1930), and from *Arbacia* (Lillie, 1913), *Strongylocentrotus* (Tyler, 1939) and *Megathura* sperm (Tyler, 1940) which become intensely motile when mixed with homologous egg water.

*Patiria*, *Pisaster* and *Astropecten* sperm, although refractory to treatment with sea water and egg water, nevertheless became intensely active when treated with isotonic hen's egg white or the serum of the lobster (*Panulirus*), fish (*Crassius*), hen, or rabbit. Furthermore, adjuvant-treated sperm of these starfish agglutinated strongly on addition of homologous egg water. *Asterias* was tested on three successive seasons. The sperm became intensely active when treated with isotonic hen's egg white. Weak agglutination sometimes occurred after addition of homologous egg water to the sperm egg white suspension. Unfortunately, the agglutination was so weak and occurred so irregularly that quantitative studies could not be made.

In *Patiria* the agglutination resulting from treatment of sperm with adjuvant and egg water was exclusively head to head. Each clump consisted of a central mass of sperm heads tightly bound together, and a peripheral region of free tails which projected out radially from the central mass of heads. *Patiria* thus differs from *Megathura*, since sperm of the latter agglutinate tail to tail as well as head to head (Tyler, 1940). The clumped *Patiria* sperm soon became immobile even though the free sperm remained active for an hour or more. The spontaneous reversal of agglutination so characteristic of the sea urchin occurred to a limited extent only after the free sperm had become inactive.

#### PROPERTIES OF PATIRIA FERTILIZIN

Fertilizin may be defined by the following properties: (1) it combines with (but does not necessarily agglutinate) sperm, (2) it is highly specific in this reaction, and (3) it is obtained primarily from eggs. Studies on the role of egg water in the agglutination of treated sperm show that *Patiria* egg water has these properties.

*Absorption of Patiria egg water by sperm.* A direct combination between sea urchin fertilizin and sperm may be demonstrated by absorption of egg water with sperm, or by neutralization of egg water with appropriate sperm extract (Lillie, 1913; Frank, 1939). Similarly, it may be shown that sperm-absorbed *Patiria* egg water will no longer agglutinate treated sperm. Indeed, complete exhaustion of the egg water may be attained even in the absence of adjuvant. In a typical experiment 20 drops of *Patiria* egg water were mixed with 22 drops of concentrated (25-50 per cent) *Patiria* sperm. The mixture was set aside to allow for reaction. Twenty drops of the same egg water were mixed with 22 drops of sea water to serve as a control. After centrifugation the fluid of both tubes was titrated with adjuvant-treated (0.5-1 per cent) sperm. The undiluted absorbed egg water did not agglutinate the sperm, whereas the control unabsorbed egg water clumped the sperm

even at a dilution of  $\frac{1}{256}$  of full strength. Other controls showed that sperm without adjuvant were not agglutinated by control or absorbed egg water, or by the adjuvant (*Patiria* sperm-absorbed *Panulirus* serum). Thus a substance (fertilizin) is present in *Patiria* egg water which will combine with specific sperm independently of the adjuvant. The adjuvant is required only for agglutination.

*Specificity of starfish fertilizin.* The reaction between sperm and fertilizin is characterized by a high order of specificity (Tyler, 1940). Cross tests between *Patiria*, *Pisaster* and *Astropecten* sperm and fertilizin show that these starfish are not exceptional in this respect. Sperm suspensions of the three species were treated with *Patiria* sperm-absorbed *Panulirus* serum, and then cross tested with the egg waters of the three species. The data are given in Table I.

TABLE I  
*Specificity of Patiria, Pisaster and Astropecten egg waters*

|                                   |                                     | <i>Patiria</i><br>sperm | <i>Pisaster</i><br>sperm | <i>Astropecten</i><br>sperm |
|-----------------------------------|-------------------------------------|-------------------------|--------------------------|-----------------------------|
| <i>Patiria</i><br>egg water +     | adjuvant                            | +++                     | ±                        | —                           |
|                                   | sea water                           | ±                       | —                        | —                           |
| <i>Pisaster</i><br>egg water +    | adjuvant                            | —                       | +++                      | —                           |
|                                   | sea water                           | —                       | ++                       | —                           |
| <i>Astropecten</i><br>egg water + | adjuvant                            | —                       | —                        | ±                           |
|                                   | sea water                           | —                       | —                        | —                           |
|                                   | adjuvant<br>+<br>sea water          | —                       | —                        | —                           |
|                                   | <i>Patiria</i><br>sperm supernatant | —                       | —                        | —                           |

It will be seen that *Patiria* and *Pisaster* egg waters agglutinated only homologous sperm. Thus the species specificity rule holds for these two forms. In this experiment *Pisaster* egg water clumped homologous untreated sperm. This reaction did not occur with predictable regularity. The reaction between *Astropecten* egg water and homologous sperm was doubtful. This may be ascribed to neutralization of the *Astropecten* egg water by the *Patiria* sperm supernatant present in the adjuvant solution. The relationship here is somewhat involved. With the exception of the reaction between *Patiria* sperm supernatant and *Astropecten* egg water, the reactions were species specific.

*The source of Patiria fertilizin.* Only egg water prepared from suspensions of *Patiria* eggs possessing their normal gelatinous coats agglutinated species sperm in the presence of the adjuvant. Blood from female animals did not have this effect. Thus it may be concluded that a specific substance is obtained from starfish eggs which will react with and under certain conditions agglutinate species

sperm. This then gives clear and direct support to Tyler's (1941) view that fertilizin exists in the *Patiria* egg water.

### THE NATURE OF PATIRIA FERTILIZIN

Tyler (1941) concluded that *Patiria* fertilizin was univalent for combining groups complementary to sperm. It follows from the lattice theory that such univalent fertilizin must become multivalent to agglutinate the sperm. The adjuvant should then convert the natural univalent *Patiria* fertilizin to a multivalent, agglutinating form. However, another possibility in accord with the lattice theory is that the fertilizin is multivalent but the sperm is normally univalent. The results of the following experiments favor this latter view.

*Effect of ultraviolet light on Patiria fertilizin.* Sea urchin fertilizin can be converted to the univalent form by proper exposure to heat, enzymes, x-radiation and ultraviolet light (Tyler, 1941; Metz, 1942). Such treated fertilizin will not agglutinate sperm but it will combine with sperm rendering the sperm unagglutinable by untreated fertilizin. To test for the possibility of a similar action, *Patiria* fertilizin was exposed to ultraviolet irradiation. It was found that irradiated *Patiria* fertilizin will not agglutinate adjuvant-treated homologous sperm, and normal fertilizin will not subsequently agglutinate the sperm that has been treated with irradiated fertilizin. Thus it is possible that the natural fertilizin is multivalent and the irradiated material is true univalent fertilizin. The data from a typical experiment are given in Table II.

TABLE II

*Destruction of agglutinating power of Patiria fertilizin by ultraviolet light and agglutination inhibiting properties of this fertilizin*

| Solution                           | Irradiated fertilizin | Control fertilizin | Irradiated fertilizin + control fertilizin | Irradiated sea water + control fertilizin |
|------------------------------------|-----------------------|--------------------|--|---|
| Reaction of adjuvant-treated sperm | —                     | ++++               | —  | ++++                                      |

Two stender dishes each containing 5 cc. of a *Patiria* fertilizin solution and one dish containing 5 cc. of sea water were irradiated for 220 minutes. The control fertilizin sample was screened from the ultraviolet light by a "noviol C" filter. After the irradiation the control and irradiated fertilizin samples were tested for agglutinin activity by mixing 2 drops of hen's egg white treated sperm (1%) with 2 drops of each fertilizin solution. At the same time 2 drops each of the sperm and irradiated sea water were mixed. It will be seen that the irradiated fertilizin was inactive whereas the control strongly agglutinated the sperm. After this examination one drop of unirradiated test fertilizin was added to the irradiated fertilizin-adjuvant-sperm mixture and one drop to the irradiated sea water-adjuvant sperm. In this test for inhibition of agglutination it will be seen that sperm treated

with irradiated fertilizin did not agglutinate upon subsequent addition of normal fertilizin, whereas the sperm treated with irradiated sea water reacted strongly.

*Agglutination of adjuvant-free Patiria sperm by fertilizin.* More definite evidence for the multivalent nature of starfish fertilizin was obtained from a study of the effect of adjuvant on sperm. Adjuvant was added to *Patiria* sperm and then removed. Such adjuvant free sperm agglutinated on addition of natural fertilizin. Twenty drops of 0.5 per cent sperm were mixed with 10 drops of isotonic hen's egg white. A control sample consisted of 20 drops of 0.5 per cent sperm plus 10 drops of sea water. Both samples were centrifuged and the packed sperm was resuspended in 20 drops of sea water. Two drops of sperm from each were tested with *Patiria* fertilizin. The control suspension did not react whereas the sperm centrifuged from the egg white agglutinated moderately. The suspensions were recentrifuged and the supernatants tested and found free of adjuvant. The sperm masses were resuspended in 16 drops of sea water after the second centrifugation and tested. The control sperm did not react to fertilizin whereas the sperm previously treated with adjuvant agglutinated weakly.

This experiment was not confirmed with *Astropecten*. *Astropecten* sperm after centrifugation from hen's egg white solution were not agglutinated by homologous fertilizin alone, although this sperm reacted strongly when both egg white and fertilizin were added.

It seems clear then that *Patiria* fertilizin will agglutinate sperm after the adjuvant has been removed from the sperm. It may therefore be concluded that the natural *Patiria* fertilizin is multivalent.

#### UNIVALENT SPERM

Evidence has just been presented to show that natural *Patiria* fertilizin is multivalent and capable of agglutinating sperm. It follows that the normal sperm is incapable of agglutination. The adjuvant must then convert the sperm to an agglutinating condition.

It seems unlikely that stimulation of the normally immobile sperm to intense activity is of any considerable importance in this adjuvant-fertilizin agglutination of *Patiria* sperm since immunological doctrine does not require motility of cells for agglutination. Thus non-motile bacteria and erythrocytes agglutinate strongly when mixed with specific antibody. Furthermore, heat killed sea urchin sperm agglutinate strongly on addition of fertilizin. However, heat killed *Patiria* sperm did not react when mixed with fertilizin and adjuvant. The deficiency of the normal sperm must then involve the antigenic structure of the cell surface. For agglutination to occur the area of the sperm surface containing groupings complementary to fertilizin must be rather extensive. If this region of the sperm surface were limited in extent and contained but a few or even a single antifertilizin group, the sperm could be considered "univalent" for this particular antigen. Such sperm should not agglutinate when mixed with complementary agglutinin (fertilizin). At best only two or three sperm could clump together. This condition is occasionally observed when untreated *Patiria* sperm and fertilizin are mixed. It has been described as the "granular" reaction.

*Absorption of Patiria fertilizin by treated and normal sperm.* If normal *Patiria* sperm are "univalent" with respect to exposed antifertilizin groups, the cells must

be made multivalent before they can be expected to agglutinate. The adjuvant is believed to effect such a conversion to the multivalent form by "exposing" latent or unreactive antifertilizin present on or near the sperm surface. Treated sperm then should bind more fertilizin than the normal "univalent" sperm. One of three absorption experiments demonstrating this is recorded in Table III.

TABLE III

*Absorption of fertilizin by sea water and egg white treated Patiria sperm*

| Absorbing Mixtures |                    |                    |                    |  |
|--------------------|--------------------|--------------------|--------------------|--|
| Tube I             | Tube II            | Tube III           | Tube IV            |  |
| 0.5 cc. sea water  | 0.5 cc. egg white  | 0.5 cc. egg white  | 0.5 cc. sea water  |  |
| 0.5 cc. fertilizin | 0.5 cc. fertilizin | 0.5 cc. fertilizin | 0.5 cc. fertilizin |  |
| 0.5 cc. sperm      | 0.5 cc. sperm      | 0.5 cc. sea water  | 0.5 cc. sea water  |  |

| Titration of absorbed fertilizin solutions |        |         |          |         |
|--|--------|---------|----------|---------|
| Dilution of absorption supernatant         | Tube I | Tube II | Tube III | Tube IV |
| 1/2  | +++    | —       | ++++     | ++++    |
| 1/4  | ++     | —       | ++++     | ++++    |
| 1/8  | +      | —       | ++++     | ++++    |
| 1/16                                       | +      | —       | +++      | +++     |
| 1/32                                       | +      | —       | +++      | +++     |
| 1/64                                       | +      | —       | ++       | ++      |
| 1/128                                      | +      | —       | ++       | ++      |
| 1/256                                      | —      | —       | ++       | ++      |
| 1/512                                      | —      | —       | +        | +       |
| 1/1024                                     | —      | —       | +        | +       |
| 1/2048                                     | —      | —       | —        | —       |

Four absorption tubes were prepared as indicated in the table. Fifty per cent sperm was used in the absorption and raw isotonic hen's egg white was employed as adjuvant. The tubes were refrigerated for nine hours to allow for complete reaction, and then centrifuged. The supernatants were then titrated for fertilizin with one per cent treated sperm. In absorption tubes III and IV sea water was substituted for the sperm added to tubes I and II. No adjustment was made in the titration for the volume of absorbing sperm removed from I and II by centrifugation. This is justified since the titration was made on a comparative basis and tubes III and IV represent controls for neutralization of fertilizin by egg white. Furthermore, the error in absolute values introduced by this involves something less than  $\frac{1}{8}$  of a dilution and therefore is well within the error of the method. Likewise, no adjustment was made in the supernatant of tube I for the adjuvant present in the absorption supernatant of tube II. Such adjustment was apparently unnecessary since the titers of the control tubes III and IV were the same (1024). The titers of these tubes also show that the egg white does not neutralize fertilizin. Comparison of tubes I and III shows that the sea water-sperm mixture caused an 8- to 64-fold drop in fertilizin concentration. However, in tube II (titer 0)

the adjuvant-sperm mixture completely exhausted the fertilizin. The striking difference in the titers of tubes I and II (128 and 0 respectively) demonstrates clearly that treated sperm has a greater fertilizin binding capacity than normal sperm.

#### EFFECT OF THE ADJUVANT ON THE FERTILIZING POWER OF PATIRIA SPERM

Since the adjuvant increases the fertilizin binding power of sperm and also the motility of these cells, it seemed likely that treated sperm would be unusually effective in fertilization. Several experiments comparing the treated and normal sperm in this respect showed this to be the case. The results of one such experiment are given in Table IV.

TABLE IV

*The effect of hen's egg white on the fertilizing power of Patiria sperm*

| Sperm dilution | Egg white treated sperm | Sea water treated sperm | Egg white + Patiria eggs | Sea water + Patiria eggs |
|----------------|-------------------------|-------------------------|--------------------------|--------------------------|
|                | % cleavage              | % cleavage              | % cleavage               | % cleavage               |
| 1/2            | 94% (75)*               | 38% (95)*               | 0.7% (152)*              | 0.0% (118)*              |
| 1/4            | 95% (66)                | 0% (58)                 |                          |                          |
| 1/8            | 89% (45)                | 7.4% (54)               |                          |                          |
| 1/16           | 88% (50)                | 6.8% (74)               |                          |                          |
| 1/32           | 89% (53)                | 2.0% (65)               |                          |                          |

\* Total number of eggs counted.

A fresh one per cent sperm suspension was divided into two parts. One part was diluted serially (in twofold steps) with boiled isotonic hen's egg white. The other part was diluted similarly but with sea water. Sperm dilutions are given as the dilution of one per cent sperm added to the eggs. One drop of each sperm suspension was added to twelve drops of *Patiria* eggs in 6 cc. of sea water. To control for parthenogenesis one drop of egg white was added to one dish of eggs and a drop of sea water to a second dish. The eggs were examined for cleavage three hours after addition of sperm.

Although the number of eggs counted was small it can readily be seen that the egg white treatment greatly increased the fertilizing power of the sperm. Even at the lowest dilutions the treated sperm was twice as effective as the untreated cells. At high dilutions the treated sperm fertilized nearly 90 per cent of the eggs whereas the normal sperm fertilized less than 10 per cent. Gray (1915) has reported a similar result with alkali treated *Asterias glacialis* sperm.

#### SPECIFICITY OF THE ADJUVANT

Although no exhaustive search was made for different sources of adjuvant, a number of unrelated preparations were encountered which stimulated *Patiria* sperm and rendered it agglutinable by fertilizin. These preparations included *Panulirus*, rabbit, fish (*Crassius*), and hen sera, and hen's egg white. Thus the source of the adjuvant is not highly specific.

## PROPERTIES OF THE EGG WHITE ADJUVANT

The adjuvant action can not be attributed to the high pH of raw egg white (Needham, 1931) since the material is active at sea water pH. Therefore, preliminary attempts were made to characterize an "active principle" in the hen's egg white. The agent is quite heat stable. Its activity was retained even after several hours at 100° C. In fact heating increased the activity of the egg white several fold. Ultraviolet light had a similar effect. This suggests the release of inactive bound agent. The "active principle" was quite nondialyzable both before and after heating. It was soluble in saturated ammonium sulfate, but insoluble in strong acetone and alcohol. Thus it is probably neither ordinary protein nor lipid.

## DISCUSSION

From the evidence presented it is concluded that fertilizin is obtained from *Patiria* eggs, and that this fertilizin, although it does not agglutinate normal sperm, is a multivalent agglutinin that reacts with the normal sperm. It is further believed that the exposed antifertilizin of normal *Patiria* sperm is limited to a small area of the sperm surface and contains only a few or even a single combining group complementary to fertilizin. For practical purposes such sperm may be considered "univalent." It is necessary to assume that some antifertilizin is exposed on the normal sperm to explain the absorption of fertilizin by such sperm and to account for the "granular" agglutination reaction. This then is a reversal of Tyler's (1941, 1942) view. He believed that the normal *Patiria* fertilizin was "univalent" and that the sperm was multivalent.

The various adjuvant solutions stimulate the sperm to intense motility and presumably expose more antifertilizin on the sperm surface. The latter effect is believed to be the essential one in rendering the sperm agglutinable. This action of the adjuvants bears a superficial resemblance to the "transformation" of human erythrocytes by an enzyme present in certain bacterial filtrates (Thomsen effect). Any human serum will agglutinate these transformed cells. There are several important differences between the process of erythrocyte transformation (Friedenrich, 1930) and the action on starfish sperm. The transformation requires a considerable period of time (15 minutes to several hours), is irreversible, and involves a fixation and subsequent release of the transforming principle. The action on *Patiria* sperm takes place very rapidly, the process reverses slowly when the adjuvant is removed, and it involves no fixation of the adjuvant. Repeated attempts failed to show any neutralization or absorption of egg white adjuvant by sperm or sperm-fertilizin mixtures. Friedenrich (1930) believes that a new agglutinin is developed which is not present in latent or unreactive form on the normal erythrocyte. However, the case of *Patiria* sperm is more easily explained by assuming that a considerable amount of antifertilizin is in latent form on or near the cell surface.

Di Macco's (1923) "coagglutination" of sheep erythrocytes by mixtures of ricin and guinea pig serum also resembles the fertilizin-adjuvant agglutination of *Patiria* sperm. Neither ricin nor guinea pig serum alone agglutinated the sheep cells. Absorption of the separate solutions with cells failed to remove the active agents. Agglutination failed to occur if the ricin and guinea pig serum were mixed

first and the sheep cells added subsequently. Thus neither of the necessary agents reacted directly with the cells. Di Macco concluded that agglutination of sheep cells resulted from a reaction between the cells and an evanescent ricin-serum complex formed at a critical stage in the reaction between these substances. It is apparent, then, that the mechanism of the coagglutination is fundamentally different from the fertilizin-adjuvant agglutination of *Patiria* sperm.

The striking difference in fertilizing power of normal and adjuvant-treated sperm can be explained by the motility of the cells. Furthermore, this effect should be expected, regardless of motility, from the recent views of Tyler (1941). He has shown that fertilizin treatment lowers the fertilizing power of *Patiria* sperm and explained this by assuming that at fertilization a union occurs between anti-fertilizin on the sperm and fertilizin at the egg surface. If all of the sperm anti-fertilizin is bound by free fertilizin, then no reaction can occur between sperm and the surface of the egg. It follows from this that the normal univalent sperm would have much less chance of reaching the egg surface in an unsaturated condition than would the multivalent sperm. At present it is impossible to judge the relative importance of the intense motility and the multivalency of the adjuvant-treated sperm in this fertilization effect. If this increased fertilizing power should be found in species that regularly give low percentages of fertilized eggs, it might be useful for technical purposes.

#### SUMMARY

I. Starfish sperm does not ordinarily agglutinate when treated with homologous fertilizin. However, when the sperm of some species (*Patiria miniata*, *Pisaster ochraceus*, *Astropecten* sp.) is treated with certain adjuvants the cells become intensely active and agglutinate when fertilizin is added. This reaction provides a means for studying the relationship between starfish sperm and fertilizin.

II. *Patiria* sperm will combine with homologous fertilizin and remove it from solution even in the absence of the adjuvant.

III. Cross tests between *Patiria*, *Pisaster* and *Astropecten* sperm and fertilizin solutions revealed no cross agglutination reactions.

IV. It is concluded that *Patiria* fertilizin is multivalent, since irradiated fertilizin will not agglutinate treated sperm but will inhibit the agglutination of such sperm by normal fertilizin; and since normal fertilizin will agglutinate sperm which has been freed of adjuvant.

V. It is suggested that normal *Patiria* sperm possesses but a single antifertilizin combining group and that more such groups are exposed on the sperm surface through the action of the adjuvant. Experiments which show that the fertilizin binding power of sperm is increased by the adjuvant support this view.

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COCHLIOPHILUS DEPRESSUS GEN. NOV., SP. NOV. AND COCHLIOPHILUS MINOR SP. NOV., HOLOTRICHOUS CILIATES FROM THE MANTLE CAVITY OF PHYTIA SETIFER (COOPER)

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INTRODUCTION

Examination of specimens of the pulmonate snail *Phytia setifer* (Cooper)<sup>1</sup> from salt marshes bordering San Francisco Bay disclosed the presence of two closely related species of flattened holotrichous ciliates within the mantle cavity. A new genus, *Cochliophilus*, is proposed to include these ciliates, which will be described herein as *Cochliophilus depressus* gen. nov., sp. nov. and *Cochliophilus minor* sp. nov.

I wish to express my appreciation to Professor S. F. Light and Professor Harold Kirby for their interest and helpful advice during the progress of this investigation.

TECHNIQUE

*Phytia setifer* occurs under matted vegetation and debris in salt marshes and in the vicinity of brackish water ponds on the Pacific Coast of central and northern California. Material for this study was collected at several localities along the east shore of San Francisco Bay at Oakland and Berkeley.

For observation of the living ciliates the shell of the snail was carefully removed and the anterior part of the animal crushed in a drop of sea water on a slide. Fixation of the organisms for permanent preparations was accomplished by liberating them in this manner on a coverglass and then dropping the coverglass smear-side down onto the surface of the fixative in a Petri dish.

Staining with iron hematoxylin gave good results following the fixatives of Schaudinn, Champy, Bouin, and Heidenhain ("susa"). For a study of the ciliary system the method devised by Bodian (1936, 1937) for impregnation with activated silver albumose (protargol) was used after fixation in Hollande's cupric-picroformol mixture. The Feulgen nuclear reaction was tried with success on material fixed in a saturated aqueous solution of mercuric chloride with 5 per cent of glacial acetic acid.

DESCRIPTION OF SPECIES

There is no agreement among protozoologists in regard to the orientation for descriptive purposes of compressed ciliates in which the cytostome is situated along the margin of the flattened body or displaced to the surface opposite that in

<sup>1</sup> Dall (1921) has implied that the species described by Cooper (1872) is distinct from *Phytia myosotis* (Drap.) of Europe and the Atlantic Coast of North America. No conclusive evidence has been presented to support or to refute this contention.

contact with the substrate. Hentschel (1924), writing of *Entodiscus* (*Cryptochilum*) *borealis*, stated that "since convention dictates that the side on which the mouth is situated shall be called ventral, we must say that the animal is flattened from side to side." Reichenow (1927-29) applied this scheme to *Conchophthirus*, as did also Kahl (1931, 1934) and Raabe (1932, 1934b).

De Morgan (1925), in his description of *Kidderia* (*Conchophthirus*) *mytili*, considered the concave under-surface to be ventral and the position of the cytostome to be lateral. Kidder (1933b) recognized the oral surface of *Kidderia mytili* as the "physiological ventral surface," but for purposes of clearness accepted De Morgan's plan of orientation. In the present paper I will follow De Morgan and Kidder in referring to that surface of the body most often found in contact with the substrate as ventral. The lateral margin on which the cytostome is situated will be referred to as the oral margin, and the opposite side as the aboral margin.

*Cochliophilus depressus* gen. nov., sp. nov. (Figs. 1 and 2)

The body outline as seen from the dorsal or ventral aspect is ovoid, often somewhat truncate at the posterior end. A view from the oral or aboral margin shows this ciliate to be much flattened, the ventral surface being slightly concave and the dorsal surface convex. In some individuals the curvature of the dorsal surface appears to be less regular than in others.

Twenty living individuals taken at random ranged from 70  $\mu$  to 107  $\mu$  in length and from 47  $\mu$  to 77  $\mu$  in width, averaging about 93  $\mu$  by 63  $\mu$ . The thickness varied from 11  $\mu$  to 16  $\mu$ . The relation of the length to the width is not the same in all specimens. Fixation of the organisms on coverglasses produced some shrinkage and frequently also distortion of shape due to compression.

The elongated peristomal area is situated in the posterior fourth of the body. Specialized ciliary elements which will be described presently extend from the anterior end of the peristomal indentation to the cytostome. That part of the peristomal area lying posterior to the cytostome is naked.

A well-defined pharynx is not present. I prefer to regard the irregular tubular structure which passes from the cytostome into the cytoplasm as the gullet. This gullet is difficult to see in living individuals, but in fixed material is demonstrable following staining in iron hematoxylin. As it approaches the macronucleus the gullet widens out and its boundaries become inexact.

A thin pellicle covers the body. Flexure of the pellicle in this ciliate is rarely noted, and then only when the animal comes in contact with solid obstructions in its path of movement. Trichocysts are absent.

The cilia of the body are disposed in 52 to 56 longitudinal rows and beat metachronously. The cilia on the dorsal and ventral surfaces are somewhat longer than those along the margin. The ventral cilia are thigmotactic, but not strongly so. On the ventral surface at the anterior end is a transverse suture (anterior field) from which the ventral rows of cilia extend backward, and from which the dorsal rows curve upward and continue posteriorly. Most of the dorsal rows converge in a characteristic pattern towards the posterior end. A definite unciliated area is evident between the longer dorsal rows and the ventral rows which curve upward a short distance over the posterior end.

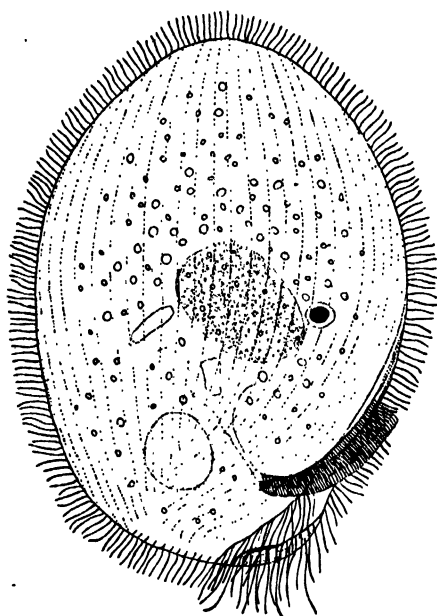


FIGURE 1. *Cochliophilus depressus* gen. nov., sp. nov. Dorsal aspect. Heidenhain's fixative ("susa")-iron hematoxylin. Drawn with aid of camera lucida.  $\times 900$ .

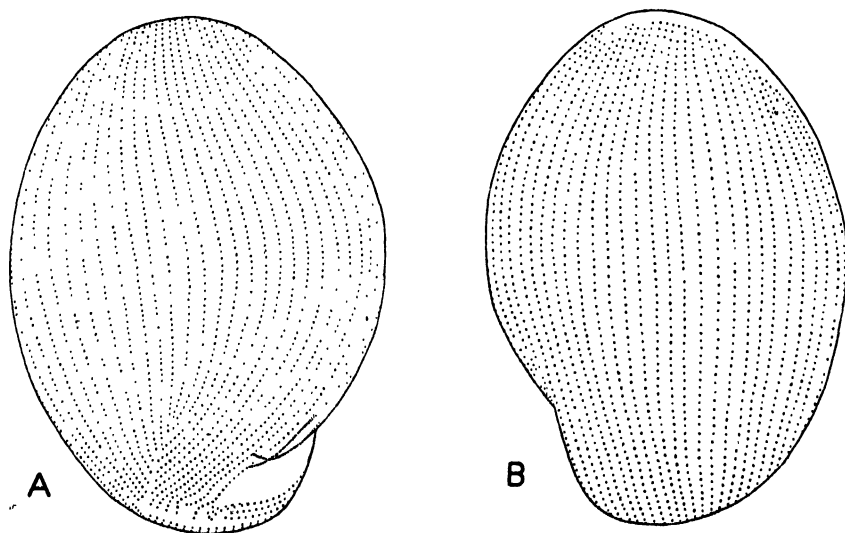


FIGURE 2. *Cochliophilus depressus* gen. nov., sp. nov. Distribution of ciliary rows. Hollande's fixative-protargol. Drawn with aid of camera lucida. A. Dorsal aspect. Though distorted somewhat due to compression, this individual shows well the arrangement of ciliary rows entering the peristomal indentation.  $\times 670$ . B. Ventral aspect.  $\times 950$ .

Three ventral rows of cilia close to the oral margin turn dorsally near the end of their course to delimit the naked part of the peristomal area posteriorly. The first of these rows is ordinarily seen to ramify into an incomplete double or triple series of cilia. The post-peristomal extensions of the ventral rows and the terminal part of the dorsal row which borders the peristome above bear cilia which are two to three times as long as the peripheral cilia elsewhere on the body.

The specialized peristomal cilia arise from two series of closely-set basal granules, each of which is seen to be a continuation of two rows of peripheral cilia essentially lateral in position, lying between the three ventral rows of cilia and one dorsal row marking off the peristomal area. The cilia of the upper peristomal row are appreciably longer than those of the lower row and appear in living individuals to form a membrane-like structure which beats up and down as a unit. The cilia of the lower row are much thicker and do not beat synchronously. The activity of the peristomal cilia ceases soon after the organism is dissociated from the host.

The cytoplasm is colorless. Greenish granules appearing as highly refractile bodies are distributed through the cytoplasm. These are most numerous around the macronucleus and following fixation stain intensely with iron hematoxylin.

The macronucleus is centrally located. In outline it varies from oblong to round, and in life is conspicuous as a clear granular body surrounded by food inclusions and cytoplasmic granules. The micronucleus is greenish in color and difficult to detect in living individuals. It is easily demonstrated by iron hematoxylin or the Feulgen nuclear reaction. The micronucleus is commonly situated close to the macronucleus, between the latter and the oral margin. Upon fixation it shrinks considerably and draws away from the membrane by which it is invested.

The contractile vacuole lies in the posterior fourth of the body behind the gullet, and apparently opens to the exterior at a point between the convergence of the shorter dorsal ciliary rows. I have been unable to distinguish a permanent opening in the pellicle.

When free in water, *Cochliophilus depressus* swims actively, generally in circles and with its concave ventral surface in contact with the substrate. Occasionally, however, it follows an erratic course, rotating on its longitudinal axis. The transverse anterior field is always at right angles to the direction of movement. In the presence of pieces of tissue from the host *Cochliophilus depressus* will sometimes seek refuge among them or cling to epithelial surfaces by means of its ventral thigmotactic cilia.

I have found *Cochliophilus depressus* to be present in the mantle cavity of nearly all specimens of *Phytia setifer* which I have examined. It occurs in small numbers and is usually less common than the following species.

*Cochliophilus minor* sp. nov. (Figs. 3 and 4)

The shape of this species resembles in general that of *Cochliophilus depressus*, except that the posterior end is rather pointed, never truncate, and the dorso-ventral dimension in relation to the length and breadth is comparatively greater. In addition, the curvatures of the ventral and dorsal surfaces are more pronounced in *Cochliophilus minor*.

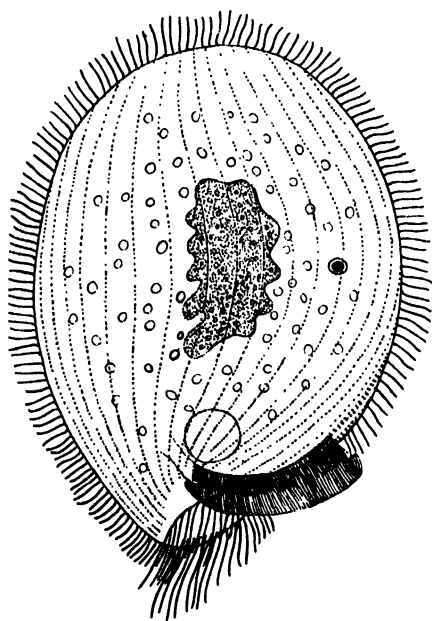


FIGURE 3. *Cochliophilus minor* sp. nov. Dorsal aspect. Heidenhain's fixative ("susa")-iron hematoxylin. Drawn with aid of camera lucida.  $\times 1250$ .

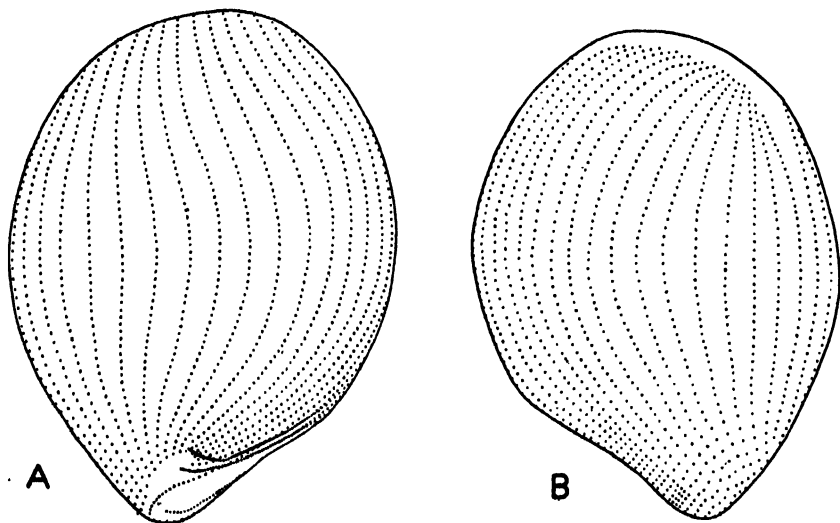


FIGURE 4. *Cochliophilus minor* sp. nov. Distribution of ciliary rows. Hollande's fixative-protargol. Drawn with aid of camera lucida. A. Dorsal aspect.  $\times 1250$ . B. Ventral aspect.  $\times 1250$ .

Twenty living individuals taken at random ranged from  $51\ \mu$  to  $80\ \mu$  in length and from  $33\ \mu$  to  $56\ \mu$  in width, averaging about  $63\ \mu$  by  $45\ \mu$ . The thickness varied from  $11\ \mu$  to  $18\ \mu$ .

The peristomal area is situated in the posterior fourth of the body. Two rows of specialized cilia extend from the anterior end of the peristomal indentation to the cytostome. That part of the peristomal area posterior to the cytostome is naked.

An irregular gullet may sometimes be traced a short distance from the cytostome, but it is not as easily discerned as the comparable structure in *Cochliophilus depressus*.

The cilia are disposed in 36 to 38 longitudinal rows and beat metachronously. The cilia on the ventral and dorsal curvatures are slightly longer than those along the margin. The ventral cilia are weakly thigmotactic. The ventral rows extend from an anterior transverse suture to the posterior tip of the body. The basal granules of most of the ventral rows come to lie farther apart towards the posterior end, while those of three or four rows near the oral margin lie closer together. The dorsal rows of cilia pass from the transverse suture over the anterior end of the body and continue backward to terminate in a conformation homologous with that found in *Cochliophilus depressus*. The posterior dorsal unciliated area of *C. depressus* has no exact homologue in this species. There exists, nevertheless, an unciliated area between the converging dorsal rows and the dorsal row bordering the peristomal area above.

One or two rows of cilia following the oral margin curve dorsally near the end of their course to delimit the naked part of the peristomal area posteriorly. These extensions and the terminal part of the most nearly lateral dorsal row on the oral side bear exceptionally long cilia.

The peristomal ciliary apparatus consists of a membrane-like structure of long, fine cilia which curves downward over a row of closely-set, rather thick cilia extending from the anterior end of the peristomal indentation to the cytostome. The membrane-like structure appears to be non-motile and to function as a funnel directing food particles into the cytostome.

The cytoplasm is colorless. Refracting cytoplasmic granules are present, but to a lesser extent than in *Cochliophilus depressus*.

The size and shape of the macronucleus are highly variable. In living as well as fixed individuals it is nearly always seen to be ramified, although ovoid or round macronuclei are occasionally noted in this species. Reorganization stages in which two or more smaller and round macronuclei are present are not infrequently met with. The micronucleus ordinarily occupies a position between the macronucleus and the oral margin. In fixed and stained preparations it is considerably shrunken.

The contractile vacuole is situated anterior to the cytostome. It opens to the exterior between the convergence of the shorter dorsal ciliary rows. I have not detected a permanent opening in the pellicle.

When separated from its host *Cochliophilus minor* swims in circles or proceeds forward rotating on its longitudinal axis. Its movements are in general slower than those of *Cochliophilus depressus*.

*Cochliophilus minor* is found in association with *Cochliophilus depressus* in the mantle cavity of *Phytia setifer*. It is usually more numerous than *C. depressus*.

## SYSTEMATIC POSITION

On the basis of certain features of the morphology of the two species of *Cochliophilus* which I have described it may be justifiable to allocate this genus to the sub-order *Thigmotricha* Chatton and Lwoff, although in view of the deficiencies of the systems of classification of holotrichous ciliates currently recognized I must defer a conclusive statement with regard to its position. The organization of the peristome of *Cochliophilus* hints its affinity with *Kidderia* Raabe, represented by *K. mytili* (De Morgan) from *Mytilus edulis*. Raabe (1936) retained *Kidderia* in the family Conchophthiridae Reichenow,<sup>2</sup> but removed to the family Thigmophryidae Chatton and Lwoff *Myxophyllum* and *Conchophyllum*, genera created by him to accommodate, respectively, Stein's species *Conchophthirus steenstrupi*, commensal on various terrestrial pulmonate molluscs, and *Conchophthirus caryoclada* Kidder, from the bivalve *Siliqua patula*. It is interesting to note, in passing, that a specific character of *Conchophyllum caryoclada* is its branched macronucleus, of which the macronucleus of *Cochliophilus minor* is reminiscent.

The presence of a membrane-like structure in the peristome of *Cochliophilus* could be the basis for objections to the inclusion of this genus in the *Thigmotricha*. Very similar ciliary elements have been observed, however, in certain species of the family Ancistrumidae Issel. Raabe (1932, 1934b) has stressed the presence of an undulating membrane in *Conchophthirus*, although Kidder (1934), after studying species of *Conchophthirus* from fresh water mussels in this country, was unable to corroborate Raabe's findings, and suggested that Raabe may have mistaken the fibers of the peristomal basket for an undulating membrane.

*Genus Cochliophilus gen. nov.*

Diagnosis: Flattened holotrichous ciliates, ovoid in outline as seen in dorsal or ventral view. The peristomal area is elongated and is situated on the right lateral margin in the posterior fourth of the body. A membrane-like structure of fine cilia overlies a series of thick cilia extending from the anterior end of the peristomal indentation to the cytostome; that part of the peristomal area posterior to the cytostome is naked. The peripheral cilia are disposed in longitudinal rows extending from a ventral transverse suture at the anterior end of the body. The dorsal rows converge in a characteristic pattern posteriorly. Thichocysts are absent. The macronucleus is centrally located; the micronucleus is usually situated near the macronucleus, between the latter and the oral margin. The contractile vacuole opens to the exterior between the convergence of the shorter dorsal ciliary rows; no permanent opening in the pellicle is discernible. Genotype: *Cochliophilus depressus* gen. nov., sp. nov. Two species, commensal in the mantle cavity of *Phytia setifer* (Cooper).

*Cochliophilus depressus gen. nov., sp. nov.*

Diagnosis: Average size about  $93\ \mu$  by  $63\ \mu$ , the thickness being about one-sixth the length. The ciliary rows are 52 to 56 in number. The peristomal membrane-like structure is motile. The macronucleus is round or oblong. Syntypes are in the collection of the author.

<sup>2</sup> Reichenow (1927-29) was apparently the first to use the name Conchophthiridae, although Raabe credits Kahl (1931) with establishing this family.

*Cochliophilus minor* sp. nov.

Diagnosis: Average size about  $63\ \mu$  by  $45\ \mu$ , the thickness being about one-fourth the length. The ciliary rows are 36 to 38 in number. The peristomal membrane-like structure is apparently immobile, serving as a funnel directing food particles into the cytostome. The macronucleus is characteristically ramified. Syntypes are in the collection of the author.

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## THE DEVELOPMENT OF MARINE FOULING COMMUNITIES

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This paper constitutes an examination of the sedentary communities found on float bottoms and other submerged objects in Newport Harbor, California. Particular attention has been paid to the changes in composition of such communities with time.

The basic problem in the development of a sequence of communities in a limited environment is that of distinguishing between seasonal progression and true succession. Seasonal progression results fundamentally from differences in breeding seasons of various organisms. This type of development was noted at Beaufort, N. C. by McDougall (1943). Most of the organisms observed by McDougall had short life cycles and short breeding seasons. As a result, most of the organisms which settled in the winter months were dead or moribund by spring, and were replaced by organisms breeding in the latter season.

Succession, in contrast to seasonal progression, involves definite relations between organisms, Shelford (1930) has suggested the following criteria for the occurrence of succession: (1) Early forms must drop out, and be replaced by later forms, and (2) Some of the earlier forms must be essential for the establishment of the later forms. The use of the word "essential" in this connection is perhaps unfortunate. It would be nearly impossible, in most cases, to prove that one organism is essential for the establishment of another. On the other hand, the presence of one organism might well provide conditions favoring the establishment of another, and certainly such favorable conditions would suffice to insure the displacement of early settlers by later arrivals.

The phenomena of ecological succession are well known in terrestrial communities. In littoral marine communities, it has sometimes been stated that true succession does not occur, or is of little importance (Shelford, 1930; McDougall, 1943). The clearest case of succession in intertidal communities is that reported by Hewatt (1935). In the *Mytilus californianus* community characteristic of exposed rocky coasts along the entire Pacific coast of the United States, the establishment of a climactic condition requires more than two and one-half years, and involves a definite sequence of organisms. The reports of Kitching (1937), Moore (1939) and Moore and Sproston (1940) also give some indication that recolonization of intertidal rock surfaces is a slow process. It appears that the first event is ordinarily a heavy settlement of algae, and that many animal forms appear only after the plants have become established. Kitching (1937) provides evidence of a succession of algal forms on rocky intertidal ledges.

The sedentary organisms inhabiting floats, pilings, boat bottoms and similar structures have been the subject of many investigations. The literature in this field has been reviewed recently by McDougall (1943) and need not be cited ex-

tensively here. The most thorough investigations dealing with the Pacific forms are those of Coe (1932) and Coe and Allen (1937). These studies, covering a period of nine years, have provided invaluable information regarding the biology of the organisms concerned. The data reported in the current study have been accumulated between February 1943 and March 1945.

#### THE FLOAT-BOTTOM COMMUNITIES OF NEWPORT HARBOR

Field observations on float bottoms and similar structures in Newport Harbor disclosed the existence of five or six rather definite communities. For convenience, throughout this paper, these communities will be referred to by designations indicating the most abundant organisms in the community. In this way, we may designate (a) algal, (b) bryozoan, (c) *Ciona*, (d) *Styela*, (e) *Mytilus*, and (f) *Balanus* communities. These communities were not all sharply marked off, one from another, and communities intermediate in composition between algal and bryozoan, bryozoan and *Styela*, *Styela* and *Mytilus*, bryozoan and *Mytilus*, and *Ciona* and *Mytilus* have been observed. The various communities showed no relation to the position of the floats in the harbor, and indeed several different communities were found within a distance of a hundred feet on different floats. Evidence will be presented that this results from a definite succession, and that the composition of the community on any particular float bottom depends on (a) the length of time during which the float has been in the water, and in part on (b) the season during which the float was first immersed. We shall first consider the composition of the various communities.

The bottoms of floats were examined with the aid of a periscopic device involving an ordinary underwater viewing glass with a mirror attached (Fig. 1). Organisms were also removed from floats with a long-handled scraper.

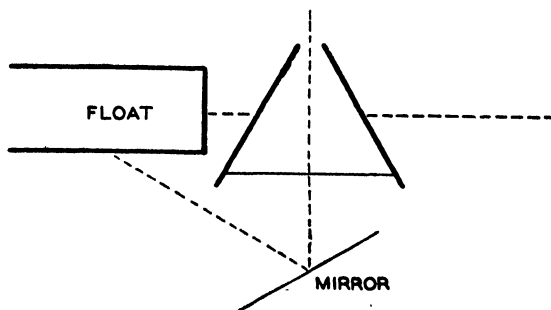


FIGURE 1. Apparatus for the examination of float bottoms.

*The algal community.* When a clean surface was placed in the bay, the first settlers were bacteria, algae, protozoans, and, during the cooler months of the year, hydroids. The algae included small sedentary diatoms which have not been identified in the present study (see Coe, 1932; and Coe and Allen, 1937), colonial diatoms of the genus *Licmophora*, and one or more species of *Ectocarpus*, notably *E. granuloides*. In addition, *Enteromorpha* sp., *Lophosiphonia villum*, and *Pterosiphonia bipinnata* were frequently noted. The sedentary protozoans in-

cluded a form similar to *Zoothamnium*, and the suctorian *Ephelota*. There were seven or eight species of hydroids; these were not identified, but *Obelia dichotoma* was usually conspicuous. Bryozoans were found in this community, sometimes in abundance. On float bottoms, *Bugula neritina* may be an important member of the community, and *Membranipora tuberculata* was observed in one instance on glass plates. *Euratea clavata*, a small semi-erect bryozoan, occasionally occurred in considerable numbers on glass plates. Finally, young colonies of a number of other species of bryozoans appeared after a time. These will be discussed in more detail later.

*The bryozoan community.* A good many floats supported a very heavy growth of bryozoans. The principal organisms involved were the encrusting bryozoans *Schizoporella unicornis*, *Cryptosula pallasiana*, *Rhynchozoon tumulosum* and *Holoporella aperta*. The erect bryozoans were less constant in occurrence, but were quite abundant in some cases. *Bugula neritina* was less frequent in this community than among the algae, while *Euratea clavata* was more frequently found among the encrusting bryozoans than among the algae. *Crisulipora occidentalis* and *Scrupocellaria diegensis* were usually present and often very abundant among the bryozoans. Four or five other species of erect bryozoans occurred less frequently.

Although the bryozoans by far outnumbered the other members of this community in most cases (Table VII), other organisms were often quite abundant. Notable are the serpulid worm *Eupomatus gracilis*, and the colonial amphipod *Erichthonius brasiliensis*. *Eupomatus* was almost always found, with its winding calcareous tubes, between the colonies of encrusting bryozoans. Occasionally, it was very abundant, the tubes making a more or less solid mass. *Erichthonius* was irregular in occurrence. During 1943, it did not appear in quantity, but in 1944 it was extremely abundant during July and August, the mud tubes often covering as much as half of the area of a glass plate. Coe and Allen (1937) noted a similar variation at La Jolla. The ascidians *Styela barnharti*, *Halocynthia johnsoni*, and *Ciona intestinalis*, and the mussel *Mytilus* sp. were found among the bryozoans in many cases, but since they were more characteristic of other communities, they will be dealt with later. Many crustaceans, annelids and other motile forms used the bryozoan clumps for shelter.

*The Ciona community.* The previous paragraphs have dealt with communities in which several species were abundant and the proportions of each species showed considerable variation in different communities of the same type. Most of the *Ciona* communities, in contrast, were composed almost wholly of specimens of *Ciona intestinalis*. This was particularly true during the summer and fall, when these communities were at their peak of development. Many float bottoms presented a solid mass of *Ciona*, with only a few other organisms present. These latter were usually colonial ascidians, growing on the tests of the *Ciona*, and such crustaceans and annelids as might have taken refuge among the stalks.

*The Styela community.* This was a poorly defined community, intermediate in composition between the bryozoan and *Mytilus* communities. The encrusting bryozoans noted earlier were usually present, forming a substratum for the stalks of *Styela*, while the erect bryozoans were often found among these stalks. Small specimens of *Mytilus* were often attached to the stalks in large numbers. Large

sponges, which have not been identified, were also frequently present, sometimes in such quantity as to dominate the community. It might indeed be preferable to refer to a *Styela*-Sponge community.

*The Mytilus community.* *Mytilus* was without question the most abundant dominant on the float bottoms in Newport Bay during the period of this study. This has not always been the case, according to reliable observers (G. E. MacGinitie, A. M. Strong, personal communications); during several previous years, *Mytilus* has not been abundant in the bay. The exact identity of this mussel remains in doubt. It is probably the same form which has been recorded infrequently from this area as *M. edulis*. However, conchologists are not entirely agreed that this is the proper designation. It is certainly not *M. californianus*. The *Mytilus* communities sometimes were observed on a substratum of old and badly decayed bryozoans; at other times they were attached directly to the float bottom. Old specimens of *Styela* or *Ciona* were often present among the mussel clumps, and various types of sponge were often quite abundant.

*The Balanus community.* Communities in which *Balanus* is the dominant organism were not observed on float bottoms in Newport Harbor, although they are frequently observed on experimental surfaces exposed in the open sea at La Jolla. Indeed, *Balanus tintinnabulum* is probably the principal dominant at La Jolla (Coe, 1932). One experimental panel exposed at this laboratory developed a *Balanus* community comparable to those observed at La Jolla, however.

#### CHANGES IN FLOAT-BOTTOM COMMUNITIES

Eight floats, all located along the mainland side of the channel between Balboa Island and Corona del Mar, and within a distance of 100 yards of one another, were selected in September of 1944, and kept under observation for a period of six months. The results of this study are presented in Table I. At intervals of about one month, the bottom of each float was examined with the viewing glass, and samples of the population removed by hand and with the scraper for later examination in the laboratory.

Float number one had been immersed in the bay for only about one week previous to the first examination. It had at that time (Sept. 21) a typical algal community, with a few specimens of *Bugula*. In October, examination showed increased numbers of *Bugula*, and a few small colonies of other erect bryozoans. In November, *Bugula* and the encrusting bryozoan *Holoporella* had displaced the algae, and a number of small specimens of *Ciona* were present. The float was then covered with a typical bryozoan community. During the remainder of the period, until March, the encrusting bryozoans continued to increase in numbers and size.

Floats 2 and 3 supported typical bryozoan communities in September. In addition to the bryozoans, a number of specimens of *Ciona* were observed, and several small *Mytilus*. During the period of observation, *Mytilus* grew at the expense of the bryozoans and ascidians, becoming very abundant in December, and largely dominating the community by February. The two float populations were very similar in composition in September, but the presence of *Styela* on float 3 in October appears to have favored the earlier establishment of *Mytilus* on this float. The presence of sponges on this float may also be related to *Styela*. Float 4, in Sep-

TABLE I  
Changes in composition of float-bottom populations, September 1944 to March 1945

| Float number | September 21, 1944                                 | October 23, 1944                      | November 22, 1944                     | December 21, 1944                 | February 13, 1945               | March 26, 1945                  |
|--------------|--|---------------------------------------|---------------------------------------|-----------------------------------|---------------------------------|---------------------------------|
| 1            | Dominants<br>Algae                                 | Algae                                 | Bugula<br>Holoporella                 | Bugula<br>Holoporella             | Encrusting Bryozoans            | Encrusting Bryozoans            |
|              | Sub-dominants                                      |                                       | Ciona                                 | Ciona                             |                                 |                                 |
|              | Influents<br>Bugula                                | Bugula                                | Encrusting Bryozoans<br>Eupomatus     | Encrusting Bryozoans<br>Eupomatus | Scrupocellaria<br>Hydroids      | Scrupocellaria<br>Hydroids      |
| 2            | Dominants<br>Encrusting Bryozoans<br>Eupomatus     | Encrusting Bryozoans<br>Eupomatus     | Encrusting Bryozoans<br>Eupomatus     | Mytilus<br>Encrusting Bryozoans   | Mytilus<br>Encrusting Bryozoans | Mytilus<br>Encrusting Bryozoans |
|              | Sub-dominants<br>Ciona Bugula<br>Scrupocellaria    | Ciona Bugula<br>Scrupocellaria        | Ciona<br>Mytilus                      | Ciona<br>Erect Bryozoans          | Erect Bryozoans                 | Erect Bryozoans                 |
|              | Influents<br>Colonial Ascidians<br>Mytilus Sponges | Colonial Ascidians<br>Mytilus Sponges | Colonial Ascidians<br>Sponges         | Colonial Ascidians                |                                 |                                 |
| 3            | Dominants<br>Encrusting Bryozoans<br>Eupomatus     | Encrusting Bryozoans<br>Eupomatus     | Encrusting Bryozoans<br>Eupomatus     | Encrusting Bryozoans<br>Mytilus   | Mytilus                         | Mytilus                         |
|              | Sub-dominants<br>Ciona Bugula<br>Scrupocellaria    | Ciona Styela<br>Scrupocellaria        | Styela<br>Mytilus                     | Sponges                           | Sponges                         | Sponges                         |
|              | Influents<br>Colonial Ascidians<br>Mytilus Sponges | Colonial Ascidians<br>Mytilus Sponges | Colonial Ascidians<br>Sponges         | Colonial Ascidians                |                                 |                                 |
| 4            | Dominants<br>Encrusting Bryozoans                  | Mytilus<br>Ciona                      | Mytilus                               | Mytilus                           | Mytilus                         | Mytilus                         |
|              | Sub-dominants<br>Ciona<br>Mytilus                  |                                       | Ciona                                 | Encrusting Bryozoans              |                                 |                                 |
|              | Influents<br>Colonial Ascidians<br>Erect Bryozoans | Colonial Ascidians<br>Erect Bryozoans | Colonial Ascidians<br>Erect Bryozoans |                                   |                                 |                                 |



tember, had a population similar to that observed on float 2 in November, with relatively large numbers of *Mytilus* and *Ciona* on a bryozoan substratum. Within a month, *Mytilus* had largely displaced the bryozoans, and within three months, *Ciona* had also disappeared.

The *Ciona* community of float 5 remained virtually unchanged from September to February. By this time, however, the *Ciona* began to show signs of deterioration. They were heavily covered with algae and hydroids, and had many small *Mytilus* about their bases. In a few places, the ascidians had fallen from the float, to be replaced by encrusting bryozoans. In March, this change had progressed so far that *Mytilus* and the bryozoans could be regarded as the dominant organisms.

Floats 6 and 7 supported two types of sponge-*Styela* communities. These were rather rapidly displaced by *Mytilus*, however. Float 8 represented a well-developed *Mytilus* community and showed no change in composition during the six months of regular observation.

These observations suggest strongly that succession is operating here. The algal community is replaced by the bryozoans, and these in turn by *Mytilus*. *Ciona* and *Styela* communities are likewise replaced by *Mytilus*, but the *Mytilus* community is relatively stable. Further information bearing on this conclusion is available from the experimental studies to be reported in the next section.

#### EXPERIMENTAL OBSERVATIONS WITH GLASS AND METAL SURFACES

Experimental observations were made using glass plates, and supplementary information was available from a series of aluminum panels immersed for another purpose. The fact that the changes observed on the glass plates were entirely similar to those observed on wooden floats and metal plates suggests that the changes reported here are not dependent on the nature of the submerged surface. Coe (1932) and Coe and Allen (1937) concluded that the seasonal variations in abundance of populations or of different groups of organisms were the same on glass, concrete, and wood surfaces. They did find significant differences in the numbers and types of organisms on the different surfaces, however.

The glass panels used were four by nine inch rectangles of ordinary window glass in most cases; in a few experiments three by five inch panels were used. The metal plates were five by eight inch rectangles of aircraft aluminum (Alclad ST-37). The glass panels were at first exposed in a horizontal frame (Fig. 2) of redwood weighted with concrete. The frame was suspended from the laboratory pier, situated in the entrance channel to Newport Harbor about one-half mile from the outer end of the jetties protecting the harbor entrance. A rapid tidal flow passes this point twice daily, carrying with it abundant larvae from both the quiet-water fauna of the harbor and the open shore fauna of the jetties and adjacent rocks. In the second year of this study, with the glass plates, and throughout the work with the metal plates, a vertical suspension was used to facilitate handling of larger numbers of plates. The plates were suspended in slotted redwood crates, with a distance of one inch between plates. As the growth on the plates became heavier, this distance was increased to two inches. The plates were always suspended one or two feet below the level of the lowest tides.

All of the plates were examined regularly at intervals of two weeks, and then returned to the bay. A count was made, in most instances, of the numbers of each of the larger species on one surface (always the same for any plate). An estimate was also made of the area covered by each of the more abundant types of organism. Usually, this was done by a direct count of ten or more low-power microscopic fields distributed over the surface. When a plate was finally removed from the water, the organisms were carefully removed, sorted and weighed.

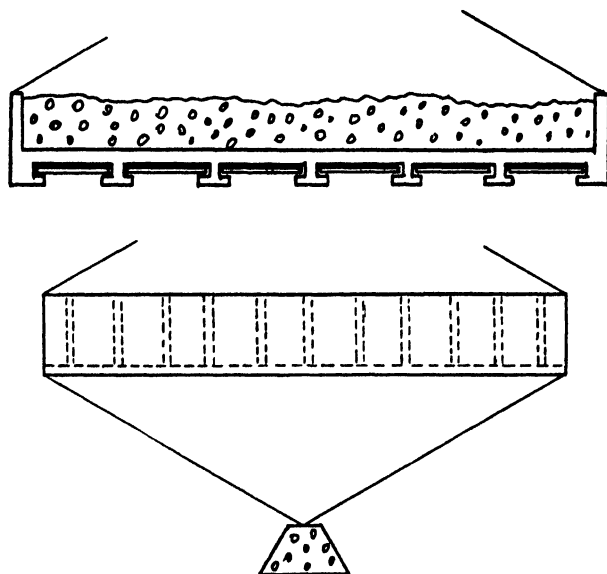


FIGURE 2. Horizontal and vertical suspension of panels.

The development of the algal community, and its transition to the bryozoan community could be followed very well on these plates. The first settlers were bacteria, diatoms, protozoans and, in the cooler months, hydroids. These were followed by the multicellular algae, especially *Ectocarpus*.

In the first months of this study it was observed that the larvae of bryozoans usually settled on the plates in quantity only after the second week of exposure, and sometimes did not settle until the fourth to sixth week. In order to verify this observation, careful counts were made during 1944 of the number of bryozoan colonies on each plate at two-week intervals. In this way, the minimum number of new settlers during any two-week period could be determined. Data obtained in this way are tabulated in Tables II, III, and IV for encrusting bryozoans, erect bryozoans and *Eupomatus*. The tabulation for the erect bryozoans omits the figures for the small semi-erect *Eucratea clavata*; representatives of this species settled in great numbers at irregular intervals, showing a behavior in this respect which was not at all comparable to the settlement of the other forms. The colonies were, moreover, rather short lived, dying often within a month of the original settlement.

During the first two weeks of exposure of any plate, the number of bryozoan and tubeworm settlements was usually less than during subsequent two-week periods. The preliminary period of light settlement was followed by a very heavy settlement in most cases. The growth of the early settlers, and in many cases, the large number of organisms settling during the maximal period, combined to reduce the available surface, and there was in consequence a very definite decrease in the number of organisms on the plate. By the time this decrease became evident, the plate was completely covered with bryozoans and tubeworms.

Number of new settlements of encrusting bryozoans on glass plates during successive two-week periods, 1944

[illegible]

Figure 3 represents data derived from a metal plate first exposed March 28, 1944, and shows the changes in area covered by the algae, bacteria and hydroids on the one hand, and bryozoans on the other. The major increase in area occupied by the bryozoans occurred after the period of maximum settlement; the heaviest settlement occurred between the sixth and eighth weeks, while the rapid increase in area began between the tenth and twelfth weeks. This was in part the result of the manner of growth of bryozoan colonies. The number of new zooids formed increases directly with the number of zooids composing the colony, so that the rate of growth increases exponentially until crowding prevents further increase in the size of the colony.

TABLE III

Number of new settlements of erect bryozoans (exclusive of *Eucratea clavata*) on glass plates during successive two-week periods, 1944

| Date examined | Date of original exposure |        |        |        |        |        |        |       |        |        |       |         |        |
|---------------|---------------------------|--------|--------|--------|--------|--------|--------|-------|--------|--------|-------|---------|--------|
|               | Jan 17                    | Jan 31 | Feb 14 | Feb 28 | Mar 12 | Mar 27 | Apr 27 | May 9 | June 8 | July 6 | Aug 1 | Sept 11 | Oct 10 |
| Mar 27        | 0                         | 0      | 0      | 0      | 0      |        |        |       |        |        |       |         |        |
| Apr. 8        | 4                         | 0      | 0      | 0      | 0      | 0      |        |       |        |        |       |         |        |
| Apr 26        | 11                        | 10     | 10     | 0      | 5      | 0      |        |       |        |        |       |         |        |
| May 9         | 20                        | 29     | 28     | 35     | 48     | 3      | 0      |       |        |        |       |         |        |
| May 24        | 5                         | 4      | 6      | -      | 17     | 18     | 0      | 0     |        |        |       |         |        |
| June 7        | 11                        | 18     | 7      | 22     | 14     | 17     | 5      | 10    |        |        |       |         |        |
| June 21       | 21                        | 27     | 36     | 24     | -      | 27     | 72     | 44    | 2      |        |       |         |        |
| July 6        |                           |        |        |        |        |        |        | 40    | 5      |        |       |         |        |
| July 17       |                           |        |        |        |        |        |        |       | 28     | 12     |       |         |        |
| July 31       |                           |        |        |        |        |        |        |       | 33     | 3      |       |         |        |
| Aug. 14       |                           |        |        |        |        |        |        |       | 28     | 11     | 1     |         |        |
| Aug. 28       |                           |        |        |        |        |        |        |       |        | 13     | 4     |         |        |
| Sept. 11      |                           |        |        |        |        |        |        |       |        |        | 9     |         |        |
| Sept. 25      |                           |        |        |        |        |        |        |       |        |        | 19    | 1       |        |
| Oct. 10       |                           |        |        |        |        |        |        |       |        |        | 18    | 0       |        |
| Oct. 23       |                           |        |        |        |        |        |        |       |        |        |       |         | 1      |

The length of time required for this sequence of events varied with the season of the year, but the character of the sequence did not vary. Thus, the plate exposed December 20 did not reach "saturation" with encrusting bryozoans until April, while the plate exposed May 9 had become "saturated" before the end of June (Table II). If we consider any particular two-week period, however, it is

TABLE IV  
Number of new settlements of *Eupomatus* on glass plates  
during successive two-week periods, 1944

| Date<br>examined | Date of original exposure |           |            |            |            |            |            |            |            |          |           |           |           |             |            |
|------------------|---------------------------|-----------|------------|------------|------------|------------|------------|------------|------------|----------|-----------|-----------|-----------|-------------|------------|
|                  | Dec.<br>20                | Jan.<br>5 | Jan.<br>17 | Jan.<br>31 | Feb.<br>14 | Feb.<br>28 | Mar.<br>12 | Mar.<br>27 | Apr.<br>27 | May<br>9 | June<br>8 | July<br>6 | Aug.<br>1 | Sept.<br>11 | Oct.<br>10 |
| Jan. 5           | 0                         |           |            |            |            |            |            |            |            |          |           |           |           |             |            |
| Jan. 17          | 0                         | 0         |            |            |            |            |            |            |            |          |           |           |           |             |            |
| Jan. 31          | 0                         | 0         | 0          |            |            |            |            |            |            |          |           |           |           |             |            |
| Feb. 14          | 2                         | 0         | 0          | 0          |            |            |            |            |            |          |           |           |           |             |            |
| Feb. 28          | 15                        | 4         | 0          | 0          | 0          |            |            |            |            |          |           |           |           |             |            |
| Mar. 12          | 17                        | 10        | 1          | 0          | 0          | 0          |            |            |            |          |           |           |           |             |            |
| Mar. 27          | 7                         | 1         | 6          | 2          | 0          | 0          | 0          |            |            |          |           |           |           |             |            |
| Apr. 8           | 8                         | 14        | 13         | 9          | 1          | 2          | 0          | 0          |            |          |           |           |           |             |            |
| Apr. 26          | 1                         | 1         | 10         | 2          | 4          | 2          | 0          | 0          |            |          |           |           |           |             |            |
| May 8            | 1                         | 3         | 2          | —          | 0          | 1          | 1          | 0          | 0          |          |           |           |           |             |            |
| May 24           |                           |           |            |            | 2          | —          | —          | 0          | 0          | 0        |           |           |           |             |            |
| June 7           |                           |           |            |            |            |            | 3          | 1          | 2          | 0        |           |           |           |             |            |
| June 21          |                           |           |            |            |            |            | 0          | 3          | 0          | 7        | 8         |           |           |             |            |
| July 6           |                           |           |            |            |            |            | 7          | 4          | 12         | 20       | 11        |           |           |             |            |
| July 17          |                           |           |            |            |            |            |            |            |            |          | 4         | 0         |           |             |            |
| July 31          |                           |           |            |            |            |            |            |            |            |          | 2         | 5         |           |             |            |
| Aug. 14          |                           |           |            |            |            |            |            |            |            |          | —         | 5         | 0         |             |            |
| Aug. 28          |                           |           |            |            |            |            |            |            |            |          | 14        | 39        | 15        |             |            |
| Sept. 11         |                           |           |            |            |            |            |            |            |            |          |           |           | 11        |             |            |
| Sept. 25         |                           |           |            |            |            |            |            |            |            |          |           |           | —         | 11          |            |
| Oct. 10          |                           |           |            |            |            |            |            |            |            |          |           |           | 4         | 3           |            |
| Oct. 23          |                           |           |            |            |            |            |            |            |            |          |           |           |           | 3           | 0          |

evident from Tables II to IV, that in general, the most recently exposed plates received lighter settlements of the three types of organisms concerned than did those which had been in the water somewhat longer. Evidently changes occurred following immersion which rendered the plate more suitable for settlement of bryozoans and tubeworms than was the clean surface. These changes occurred more rapidly in the warmer months.

Two experiments were performed to test this hypothesis, and to throw more light on the nature of the changes involved. ZoBell and Allen (1935) and Coe and

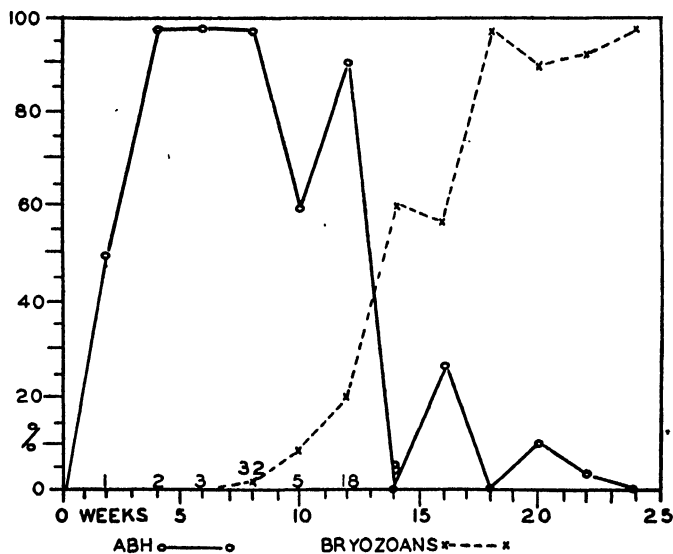


FIGURE 3. Relative areas, in per cent, covered by algae, bacteria, and hydroids (A B H), and bryozoans on an aluminum panel exposed March 28, 1944. The figures along the abscissa represent number of new settlements of bryozoans in each two-week period.

Allen (1937) have suggested that bacterial film is an important feature in the establishment of sedentary forms on a submerged surface. In the first experiment (Table V), ten three by five inch glass plates were sterilized. Two were then exposed in the bay, two were left in sterile sea water, two in a sterile solution of 0.1 per cent peptone in sea water, and two were placed in a solution of 0.1 per cent peptone in water freshly drawn from the bay. After four days, by which time a vigorous bacterial population had developed in the bay water solution, all ten

TABLE V

Settlement of organisms on pretreated glass plates, June 6-10, 1944. Duration of treatment, 4 days. Figures represent number of organisms or colonies

| Treatment:                          | First series (3 days immersion) |           |           | Second series (4 days immersion) |           |           |
|-------------------------------------|---------------------------------|-----------|-----------|----------------------------------|-----------|-----------|
|                                     | Hydroids                        | Bryozoans | Ascidians | Hydroids                         | Bryozoans | Ascidians |
| Sterile sea water                   | 43                              | 0         | 0         | 68                               | 4         | 0         |
| Sterile sea water + 0.1%<br>peptone | 53                              | 2         | 2         | 44                               | 0         | 0         |
| Bay water + peptone<br>(bacterial)  | 150                             | 2         | 2         | 174                              | 4         | 0         |
| Immersion in bay 4 days             | 53                              | 11        | 11        | 83                               | 14        | 0         |
| Sterile plate                       | 47                              | 2         | 2         | 52                               | 3         | 0         |

plates were placed in the bay. The results are presented in Table V. The hydroids evidently settled more abundantly on the bacteria-coated plates than on the others, but the bryozoans and ascidians were not influenced by the bacterial coating. Rather, they settled more abundantly on the plates which had been in the bay longest; these plates had a more abundant diatom population than did the others.

A second similar experiment was carried out in the fall, with daily observations during several weeks of exposure, and careful determinations of the bacterial and algal populations. Diatoms appeared on the plates in small numbers within the first two to four days in the bay (Table VI). For a period of two to three weeks, however, the diatoms covered less than 5 per cent of the surface. This period was

TABLE VI

Settlement of organisms on treated panels, October 21 to November 24, 1944. The figures represent the per cent of the area of one side of the panel covered by bacteria, diatoms, and protozoans respectively, and total number of organisms or colonies on one side of the panel for the larger organisms (bryozoans, annelids, ascidians).

| Duration of treatment:                                |  | 5 days |         |                             |                           | 18 days |         |                             |                           |
|---|--|--------|---------|-----------------------------|---------------------------|---------|---------|-----------------------------|---------------------------|
| Treatment:  |  | Bay    | Sterile | Sterile sea water + peptone | Fresh bay water + peptone | Bay     | Sterile | Sterile sea water + peptone | Fresh bay water + peptone |
| Days after treatment                                  | Organism                               |        |         |                             |                           |         |         |                             |                           |
| 7   | Bacteria                               | 0.3%   | 0.2%    | 0.3%                        | 1%                        |         | 1%      | 2%                          | 11%                       |
|   | Diatoms                                | 4%     | 1%      | 1%                          | 1%                        | 80%     | 0.3%    | 1%                          | 1%                        |
|   | Protozoans                             | +      | +       | +                           | +                         |         | 0.3%    | 1%                          | +                         |
|   | Bryozoans:                             |        |         |                             |                           |         |         |                             |                           |
|   | Membranipora                           | 6      | 3       | 1                           | 9                         | 0       | 1       | 0                           | 0                         |
|   | Other encrusting forms                 | 0      | 0       | 2                           | 0                         | 8       | 1       | 2                           | 3                         |
|   | Annelids                               | 0      | 1       | 0                           | 3                         | 3       | 0       | 0                           | 8                         |
|   | Ascidians                              | 1      | 0       | 5                           | 3                         | 0       | 0       | 3                           | 7                         |
| 16  | Bacteria                               | 1%     | 1%      | 2%                          | 1%                        |         | 5%      | 7%                          | 8%                        |
|   | Diatoms                                | 35%    | 11%     | 8%                          | 16%                       | 51%     | 5%      | 4%                          | 18%                       |
|   | Protozoans                             | 6%     | 1%      | 0.1%                        | 6%                        |         | 3%      | 5%                          | 6%                        |
|   | Encrusting bryozoans exc. Membranipora | 13     | 6       | 11                          | 14                        | 14      | 8       | 9                           | 17                        |
|   | Annelids                               | 1      | 2       | 2                           | 3                         | 8       | 0       | 1                           | 9                         |
|   | Ascidians                              | 2      | 2       | 9                           | 5                         | 0       | 2       | 1                           | 0                         |
|   |  |        |         |                             |                           |         |         |                             |                           |
| Time of maximum increase, days after immersion in bay | Diatoms                                | 19     | 14      | 20                          | 14                        | 19      | 16      | 14                          | 16                        |
|   | Encrusting bryozoans exc. Membranipora | 19     | 14      | 22                          | 16                        | 21      | 16      | 16                          | 16                        |

followed by a relatively sudden increase in the number of diatoms, until 25 per cent to 80 per cent of the plate was covered. The reason for this delay is not clear. During the first two to three weeks of exposure, bacteria and protozoans as well as diatoms settled on the plates. That bacteria were not concerned in the eventual diatom outburst is indicated by the fact that the bacteria-coated plates (bay water and peptone) showed no difference from the other plate in the time of the outburst. About 5 per cent of the area of the plate which had been immersed in bay water plus peptone for five days was covered with bacteria when the plate was immersed in the bay; the eighteen day plate was covered to an extent of about 11 per cent. This coating was largely lost within a few days, however. It is noteworthy that, although the time of maximum increase of the diatoms was not affected by the presence of bacteria, larger populations of diatoms eventually developed on the bacteria-coated plates than on the other plates. Algae other than diatoms were not important in this experiment; *Ectocarpus*, *Enteromorpha*, *Cladophora*, *Scytosiphon*, *Pterosiphonia* and *Lophosiphonia* were noted, but did not appear in quantity until some time after the diatom increase.

The data available from this study are not sufficient to establish a succession within the algal community. Wilson (1925), however, has reported a definite sequence, involving diatoms, hydroids and filamentous algae, on rocks at La Jolla. It is quite possible that careful study over a longer period would reveal a similar situation here.

It appears that the relatively heavy growth of diatoms on the bay water plus peptone plates is correlated with a correspondingly heavy settlement of bryozoans. Whether there is a direct causal relation between diatom growth and bryozoan settlement is uncertain. However, the maximum period of bryozoan settlement never preceded, and usually followed, the period of maximum diatom increase in the experiment of Table IV.

The encrusting bryozoan *Membranipora tuberculata*, which normally inhabits the stipes of kelp, occurred on the experimental plates only on one occasion, and remained only a short time. Most of the colonies fell from the plate within the space of a month. Unlike the other bryozoans, however, this species showed definite preference for the bacteria-coated plate.

These experiments suggest, then, that the important change which occurs on plates favoring bryozoan settlement, is the growth of diatoms. In view of this conclusion and of the fact that the bryozoan community, throughout the course of this study, has been observed to form only on surfaces previously supporting an algal growth, we may say that a definite succession is established.

The observations on the glass plates provide little evidence concerning the other communities, but two instances are worthy of mention. The development of a *Styela* community from a bryozoan community was noted in one instance, on a plate exposed horizontally in March 1943. The algal coat which developed upon exposure was displaced by bryozoans before the end of June. In September, however, specimens of *Styela* which had settled in July had become so large as to dominate the community. The remaining bryozoans gradually lost ground and fell from the plate, leaving *Styela* as the principal organism present. The fact that *Styela* was always found growing out of a substratum of old bryozoan colonies on the floats examined in the course of this study indicates that this sequence probably occurs frequently.

The second instance concerns the formation of a *Balanus* community. The plate concerned was exposed horizontally in August 1943. The algal community formed very rapidly, and in addition, within two weeks, larvae of *Eupomatus*, encrusting bryozoans, *Pecten* and *Balanus* had settled in large numbers. In the ensuing competition for space, the barnacles emerged victorious. In September, there were more than two hundred barnacles on the exposed side of the plate, covering the surface almost completely. An equal number of *Eupomatus* occupied the spaces between the barnacles, but encrusting bryozoans were not abundant. During subsequent months, however, growth of the bryozoans was continuous, and by December, the barnacles were almost completely covered by the rapidly growing bryozoans.

#### DISCUSSION AND CONCLUSIONS

In order to make a satisfactory analysis of the phenomena described in the preceding sections, it would be necessary to know (a) the breeding seasons of the organisms involved, (b) the normal duration of life of each of the important organisms, (c) the length of the free-swimming larval period in each case, and (d) the nature of the surfaces to which such free-swimming larvae will attach. We do not have such information in most cases. Nevertheless, it is possible to make some interpretations on the basis of the available information.

It was stated at the outset that the basic problem is that of distinguishing between seasonal progression and true succession. Several examples of progression were noted at Beaufort, N. C. by McDougall (1943). The organisms which settled during the winter were, for the most part, dead or moribund by spring, and were consequently replaced by organisms breeding chiefly in the spring. There is some reason to expect that seasonal progression may be less important in Newport Harbor than at Beaufort. The annual range of monthly mean temperatures at Beaufort is 23° C., from 5.5° in February to 28° in July. The annual range in Newport Harbor is only 5° C., from a low of 14.1° in February to a high of 19.2° in July. The breeding seasons of most of the organisms involved in the sequences described here extend through most of the year. Certainly algae, bryozoans and mussels have been observed to settle during every month of the two years covered by this study.

In the present study, it seems probable that the algal community, and most probably the diatoms comprising the basis of that community, provide favorable conditions for the settlement of bryozoans. Bryozoans settled in quantity only after the development of a fairly vigorous algal community. Moreover, in the experimental test described in Table VI, bryozoan settlement was definitely correlated with the settlement and growth of diatoms. There remains the possibility that some common factor favored settlement of both diatoms and bryozoans, the former remaining "dominant" only until the slower but persistent growth of the latter displaced them. It is difficult to rule out such common factors, but it appears unlikely that chemical alterations in the glass on exposure to sea water are involved. The plates used in this particular experiment had previously been immersed in the bay for two months. They were then scrubbed with a brush in tap water, wrapped in paper and sterilized in an autoclave. The experimental plates were soaked in three liters of solution for several days as noted in the table,

TABLE VII  
Weights of organisms removed from glass and aluminum panels, 1943-44  
(+ sign indicates organisms present in amounts of less than 1 gram)

| Surface:                           | Glass    |          |          |          |         |          |          |          |          |          |          |          | Aluminum  |          | G        | Al       |
|------------------------------------|----------|----------|----------|----------|---------|----------|----------|----------|----------|----------|----------|----------|-----------|----------|----------|----------|
|                                    | 4        |          |          |          | 8       |          |          |          | 20       |          |          |          | 26        | 28       | 32       | 36       |
|                                    | Mar. '43 | Apr. '43 | Dec. '43 | Mar. '43 | May '43 | July '43 | Dec. '43 | Dec. '43 | Jan. '44 | Feb. '44 | Mar. '44 | Apr. '44 | Sept. '43 | Mar. '44 | Jan. '44 | Dec. '43 |
| Duration of exposure, weeks:       | 12       | 4        | 20       | 12       | 7       | 5        | 20       | 20       | 31       | 28       | 27       | 27       | 21        | 28       | 17       | 14       |
| Date of initial exposure:          | '43      | '43      | '43      | '43      | '43     | '43      | '43      | '43      | '43      | '44      | '44      | '44      | '43       | '44      | '44      | '43      |
| Total weight of organisms, grams:  | 20       | 56       | 9        | 23       | 67      | 18       | 12       | 19       | 117      | 204      | 302      | 174      | 83        | 194      | 193      | 128      |
| Per cent of weight contributed by: |          |          |          |          |         |          |          |          |          |          |          |          |           |          |          |          |
| Algae, Bacteria, Hydroids, Debris: | 100      | 85       | 100      | 87       | 76      | 28       | 100      | 81       | 2        | 1        | 30       | 9        | 59        | 21       | 18       | 11       |
| Bryozoans                          |          |          |          | +        | 1       | 29       | +        | 5        | 57       | 54       | 34       | 35       | 30        | 63       | 27       | 66       |
| Erect forms                        |          |          | +        |          | 9       | 3        | +        | +        | 21       | 22       | 11       | 25       | 2         | 6        | 28       | 1        |
| Serpulids                          |          |          |          |          | +       | 14       | +        | +        | 10       | 2        | 1        | +        | 6         | 1        | 5        | 1        |
| Others                             |          | 8        |          | +        | 1       |          | +        | 3        | 1        | 5        | 1        | 1        | 1         | 1        | 1        | 2        |
| Crustaceans                        |          | 4        | +        |          |         | 3        | +        | 11       | 8        | 5        | 21       | 14       | 1         | 3        | 10       | 5        |
| Erichthonius                       |          |          |          |          |         | 14       |          |          | +        |          | +        | +        | 1         | +        |          | 1        |
| Balanus                            |          |          |          |          |         |          |          |          | +        |          | +        | +        |           |          |          | +        |
| Others                             |          | 3        | +        | +        | 1       | 3        | +        | +        |          | +        | +        | +        | +         |          | +        | +        |
| Pecten                             |          |          |          | +        | 10      | 6        | +        |          |          |          | +        | 1        |           |          |          |          |
| Mytilus                            |          |          |          |          |         |          |          |          |          |          |          |          |           | 3        |          |          |
| Saxicava                           |          |          |          | +        | +       |          |          |          | +        | +        | +        | 4        | +         | 1        | 8        | 8        |
| Sponges                            |          |          |          |          |         |          |          |          |          | 5        | +        |          | 1         | +        | +        | +        |
| Ascidians                          |          |          | +        | +        |         | +        |          |          | 1        | 5        | 1        | 11       |           | 1        | 2        | 5        |

while the control plates were placed directly in the bay. The same sequence of organisms is apparent on both experimental and control plates, and the periods of time involved are not significantly different.

The data in Table VII are of interest in this connection. It is apparent that the organisms listed fall into four classes: (1) Those which appear in abundance on all plates, but most abundantly on those exposed for the shortest periods (algae, etc.). (2) Those which appear only on plates exposed more than four weeks, and most abundantly on plates exposed twenty weeks or longer (bryozoans, serpulid worms). (3) Those which appear in measurable quantities only on plates exposed twenty weeks or longer, and are not abundant even on plates exposed as long as thirty-six weeks (*Mytilus*, *Saxicava*, sponges, ascidians). (4) Those which appear irregularly, without relation to the duration of exposure (annelids, *Balanus*, *Erichthonius* and other crustaceans, *Pecten*).

It is particularly significant that the dominant organisms of the primary communities involved in the sequence described earlier—viz. algae, bryozoans, ascidians and mussels—fall into separate categories on this basis, and that the sequence here is the same as that observed in the sequence of communities. It appears that the settlement of ascidians certainly and mussels probably is favored by the existence of a thriving bryozoan community.

In any event, there is no evidence that seasonal progression is involved to a significant extent in the algae-bryozoan-*Mytilus* sequence. A plate exposed in December went through the same sequence as did one exposed in March or April; the time relations varied, but the sequence did not. And in the absence of a seasonal progression, it is difficult to avoid the conclusion that true succession is involved.

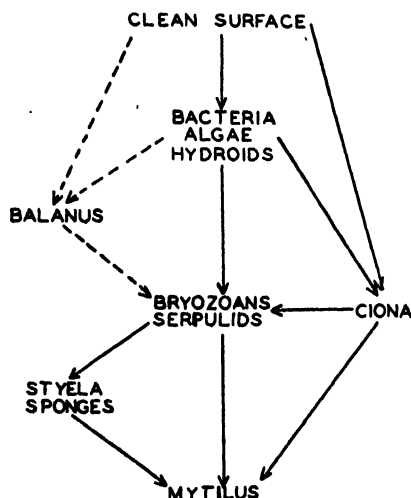


FIGURE 4. Sequence of dominant organisms on surfaces exposed in Newport Harbor.

In many studies of the life histories of sedentary organisms, estimates of the season of settling have been based on the number of new settlers on a plate exposed for a brief period. It is evident from the results reported here that such

estimates may be unreliable if succession is involved in the settlements under consideration. Thus, plates exposed for four weeks or less in Newport Harbor in the winter months would receive few or no settlements of bryozoans, despite the fact that settling larvae are present in the water during these months. It is important, therefore, that studies of this sort take into consideration the question whether succession is occurring.

With the evidence presented in this paper, we can make a number of suggestions as to the possible factors involved in the events described. The sequence is depicted in Figure 4. A newly exposed surface is first colonized by bacteria, algae and, in some seasons, hydroids. The development of these forms provides a favorable surface for establishment of bryozoan colonies, and also for the settlement of serpulid larvae. The vigorous growth of the bryozoans eventually displaces the algae and hydroids. The resulting bryozoan community in turn provides a favorable basis for the attachment of *Mytilus* larvae. The growth of the mussels effectively covers the whole surface of the bryozoan community, the members of which eventually perish from lack of food, oxygen, etc. Seasonal factors, involving the settlement of ascidian or barnacle larvae in tremendous numbers, may introduce variations into this sequence. *Ciona* may sometimes colonize a clean surface, or one covered with algae, to such an extent that the bryozoans are unable to maintain their foothold. *Styela* apparently settles only on bryozoan substrata, but may become established before *Mytilus*, and hence a community dominated by *Styela* may follow the bryozoan stage. Sponges are frequently associated with *Styela*. Both *Ciona* and *Styela* communities are eventually displaced by *Mytilus* which at present represents the climax in the float-bottom associations of Newport Harbor.

#### SUMMARY

1. The sedentary communities characteristic of float bottoms in Newport Harbor, California, are described.
2. The most important communities at present are dominated, respectively, by algae, bryozoans, *Ciona intestinalis*, *Styela* sp. and *Mytilus* sp.
3. These communities represent stages in an ecological succession.
4. The algal community appears first on freshly exposed surfaces, to be followed usually by a bryozoan community.
5. The bryozoans prominent in the bryozoan community settle more readily on surfaces supporting a vigorous growth of diatoms and other algae than on clean surfaces.
6. The community dominated by *Mytilus* constitutes the climax at present.
7. *Mytilus* has been observed to settle only on surfaces bearing a bryozoan, *Ciona* or *Styela* community.
8. The establishment of *Ciona* or *Styela* communities appears to depend in part on seasonal factors.

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# A COMPARISON OF THE EFFECTS OF CYANIDE AND AZIDE ON THE DEVELOPMENT OF FROGS' EGGS<sup>1</sup>

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Loeb's (1895) observations that the eggs of *Fundulus heteroclitus* are capable of considerable development under anaerobic conditions has since been extended to various amphibian embryos. Brachet (1934), in confirming the possibility of anaerobic development for *Rana temporaria* eggs, reported also that cyanide is similar to anaerobiosis in its effects on embryogenesis. Eggs placed in cyanide immediately after fertilization were arrested in the late blastula, but those placed in cyanide after gastrulation had begun would continue to the formation of a complete blastopore. Later stages were increasingly sensitive to cyanide. Although it has generally been thought that the arrests of development caused by cyanide are due to inhibition of the cytochrome oxidase of the Warburg-Keilin system (Keilin, 1933), it might be inferred from the recent work of Holtfreter (1943) that the repressive effects of cyanide solutions result merely from their alkalinity. It will be shown in this paper however that only post-mortem effects are influenced by the pH of the cyanide solution, the actual stoppage resulting from the presence of the toxic radical itself.

In 1936 Keilin reported in detail on another specific inhibitor of cytochrome oxidase, sodium azide ( $\text{NaN}_3$ ). On the basis of these experiments  $\text{NaN}_3$  and  $\text{NaCN}$  have in some cases been used interchangeably. Philips (1940), in comparing the developmental sensitivities to anaerobiosis of pelagic and non-pelagic fish eggs, employed both  $\text{NaCN}$  and  $\text{NaN}_3$ . He found that *Fundulus* eggs before the end of gastrulation are capable of extensive development in concentrations of both cyanide and azide which completely and almost immediately inhibit pelagic eggs. Except for the higher concentrations required in the case of  $\text{NaN}_3$  he could demonstrate no difference between the effects of the two reagents. Recently Barnes (1944) tested the same reagents on the development of *Rana pipiens*. The results with cyanide confirmed the earlier observations of Brachet (1934). While no detailed data are given, the effects of azide were apparently found to completely parallel those of cyanide, for Barnes states: "Eggs exposed to M/100  $\text{NaN}_3$  at pH 7.0 are able to develop to the gastrula stage. Gastrulation never occurs in the presence of azide." Lower concentrations (M/1000) did not stop gastrulation although the eggs developed at a slower rate.

The present authors (Moog and Spiegelman, 1942), while investigating the relation between regeneration and metabolic activity, demonstrated a specific difference between the effects of azide and cyanide on hydranth reconstitution in *Tubularia*. Azide could inhibit regeneration at concentrations which did not sensibly

<sup>1</sup> Aided by a grant from the Rockefeller Foundation.

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affect respiration whereas cyanide caused parallel depressions in rates of regeneration and of respiration. Subsequent analysis (Spiegelman and Moog, 1944) of the differential effects of these two agents on the mass and time of appearance of the new hydranth emphasized the difference in their activities.

In the fall of 1941 the authors undertook a comparison of the effects of NaCN and  $\text{NaN}_3$  on the development of *Rana pipiens*.<sup>3</sup> The results obtained disagree in certain respects with those reported by Barnes (1944). Azide was found to be completely effective in stopping morphogenesis at all stages of development, including those between fertilization and gastrulation which are not inhibited by cyanide. In an effort to discover the cause for the disagreement these experiments were repeated recently under conditions closer to those employed by Barnes. Our earlier results were confirmed and the discrepancy remains unresolved. No direct comparison with the findings of Philips (1943) is possible, not only because of the difference in material but also because the highest concentration he employed was below the one we found to give consistent inhibitions.

The results will be detailed and the difference obtained between the effects of azide and cyanide will be discussed in the light of recent findings on azide inhibitions of anaerobic synthetic processes.

#### GENERAL METHODS AND MATERIALS

Eggs of *Rana pipiens*, obtained by injection of pituitary glands, were expressed and artificially fertilized. After swelling of the jelly the eggs were cut up into small groups in 10 per cent Ringers solution adjusted to the desired pH with phosphate buffer. Stages were determined according to the schedule of Pollister and Moore (1937) and are so numbered in the present paper. The eggs were stripped from the jelly with fine forceps before being immersed in the experimental solutions.

All hydrogen ion concentrations were determined with a glass electrode after the reagents were added. Where temperature control is indicated the designated temperature was held within  $\pm 0.2^\circ \text{C}$ . Other experimental details will be found in the appropriate places of the text.

#### EXPERIMENTAL RESULTS

##### *The effects of azide and cyanide on development*

Keilin (1936) as well as subsequent investigators demonstrated the critical influence of pH on the effectiveness of azide as a respiratory inhibitor. Using the isolated Warburg-Keilin system as well as yeast cells Keilin obtained maximal effects at about pH 6.3 when the azide was used in concentrations of 0.001 and 0.002 molar. In the experiments to be described in the present section azide solutions were adjusted to pH 6.6. The concentration chosen for study was 0.005 molar, since parallel experiments on the effects on respiration (see Spiegelman and Steinbach, 1945) indicated maximal effects at this concentration on respiratory rate. The same can be said for development, for 0.005 M azide yields completely effective inhibition. All controls for the azide experiments were similarly adjusted

<sup>3</sup> These studies were carried out in the laboratories of the Department of Zoology, Columbia University, New York.

to pH 6.6. In the case of cyanide both experimentals and controls were run at pH 8.4. The controls at pH 8.4 did not differ detectably in rate of development from those at pH 6.6. Every experimental set had its own control and both were thus handled exactly the same number of times and in the same fashion. This avoided the relatively more frequent handling and examination of the controls which would have been necessary if one set of eggs were the controls for a larger number of experimentals. For convenience in observations all of the present experiments were done at 15.2° C. in a cold room. To avoid the accelerating and decelerating effects of changing temperatures during development (see Ryan, 1943) the eggs were kept at 15.2° C. in 10 per cent Ringers until they reached the stage it was desired to test. They were then transferred to the approximate solutions previously brought to the same temperature. The cyanide solutions were freshly prepared and renewed every 12 hours during the course of an experiment; the experimental solutions were kept in stacked fingerbowls, with an empty bowl covering the top member of the stack.

For the purposes of comparison with the azide experiments, the results with cyanide in the early stages are reproduced in Table I. They do not differ in essentials from those reported by Brachet (1934). Eggs placed in cyanide early in development, although delayed as compared with controls, continue to develop up to gastrulation. The later the stage at which they are subjected to cyanide the closer is the approach to gastrulation; they do not however actually begin to gastrulate. Eggs in early stage 9 will continue to segment until the cells at the vegetal pole are quite minute but will evidence no signs of dorsal blastopore lip formation. If however the invagination has already started cyanide will not immediately stop it and the eggs may proceed to the formation of a complete blastopore before ceasing activity. Later stages become increasingly sensitive to cyanide.

TABLE I

The effects of cyanide on development at pH 8.4 at 15.2° C. These experiments were done in 1941-2 on material obtained from Vermont. The numbers represent the developmental stages as described under Methods.

| Stage at start of experiment | Solution | Hours after immersion |    |    |    |    |    |    |    |    |    |     | No. of eggs |
|------------------------------|----------|-----------------------|----|----|----|----|----|----|----|----|----|-----|-------------|
|                              |          | 4                     | 8  | 12 | 18 | 24 | 35 | 45 | 55 | 75 | 95 | 120 |             |
| Uncleaved                    | 0.001 M  | 2                     | 3  |    | 6  | 8  | 8  | 8  | 8  |    |    |     | 210         |
| Uncleaved                    | Control  | 3                     | 3  |    | 8  | 8  | 10 | 11 | 12 |    |    |     | 210         |
| 3                            | 0.001    |                       | 4  |    | 6  | 7  | 8  | 8  | 9  | 9  | 9  |     | 200         |
| 3                            | Control  |                       | 6  |    | 8  | 9  | 10 | 11 | 12 |    | 16 |     | 200         |
| 6                            | 0.001    |                       | 7  |    |    | 8  | 8  | 8  | 8  | 9  | 9  | 9   | 160         |
| 6                            | Control  |                       | 8  |    |    | 10 | 11 | 12 | 12 | 13 | 16 | 18  | 160         |
| 9                            | 0.001    |                       | 9  |    | 9  |    | 9  | 9  |    |    |    |     | 200         |
| 9                            | Control  |                       | 10 |    | 11 |    | 12 | 13 |    | 16 |    |     | 200         |
| 10                           | 0.001    |                       |    | 10 |    | 10 |    | 11 |    | 12 |    | 12  | 200         |
| 10                           | Control  |                       |    | 11 |    | 12 |    | 14 |    | 16 |    | 18  | 200         |

The results obtained with azide are summarized in Table II. It is immediately evident that all stages are azide sensitive, including the early ones which are not effectively inhibited by cyanide. It might be noted that under these experimental conditions the cessation of developmental activity on immersion in azide solution is, as far as can be determined, abrupt and immediate. This was easily ascertained

TABLE II

The effect of azide on development at pH 6.6 at 15.2° C.; 1941-2, material from Vermont.  
The numbers represent the developmental stages as described under Methods

| Stage at beginning     | Solution           | Hours after immersion |          |          |          |          |          |          |          |          |          |         | No. of eggs |
|------------------------|--------------------|-----------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|---------|-------------|
|                        |                    | 4                     | 8        | 12       | 18       | 24       | 35       | 45       | 55       | 75       | 95       | 120     |             |
| Uncleaved<br>Uncleaved | 0.005 M<br>Control | 1<br>3                |          | 1<br>5   |          |          | 1<br>10  | 1<br>11  | 1<br>12  | 1<br>14  | 1<br>16  | 1<br>18 | 100<br>100  |
| 6<br>6                 | 0.005 M<br>Control |                       | 6<br>7   |          |          | 6<br>9   | 6<br>10  | 6<br>11  | 6<br>12  | 6<br>14  | 6<br>16  | 6<br>18 | 150<br>140  |
| 7<br>7                 | 0.005 M<br>Control |                       | 7<br>9   | 7<br>10  |          | 7<br>11  |          | 7<br>12  | 7<br>14  | 7<br>16  | 7<br>17  | 7<br>18 | 90<br>90    |
| 9<br>9                 | 0.005 M<br>Control | 9<br>10               |          |          | 9<br>11  |          |          | 9<br>13  |          | 9<br>16  | 9<br>17  | 9<br>18 | 110<br>110  |
| 10<br>10               | 0.005 M<br>Control |                       |          | 10<br>11 |          |          | 10<br>13 |          |          | 10<br>17 | 10<br>18 |         | 160<br>160  |
| 11<br>11               | 0.005 M<br>Control |                       | 11<br>12 |          |          |          | 11<br>14 |          | 11<br>16 |          | 11<br>18 |         | 85<br>85    |
| 12<br>12               | 0.005 M<br>Control |                       |          |          |          | 12<br>14 |          |          | 12<br>16 |          |          |         | 105<br>105  |
| 13<br>13               | 0.005 M<br>Control |                       | 13<br>14 |          |          | 13<br>16 | 13<br>17 |          | 13<br>18 |          |          |         | 120<br>120  |
| 14<br>14               | 0.005 M<br>Control |                       |          |          | 14<br>16 |          | 14<br>17 | 14<br>18 |          |          | 14<br>19 |         | 90<br>90    |
| 16<br>16               | 0.005 M<br>Control |                       |          | 16<br>17 |          |          | 16<br>18 |          | 16<br>18 | 16<br>19 |          |         | 110<br>110  |
| 17<br>17               | 0.005<br>Control   |                       |          |          | 17<br>18 | 17<br>18 | 17<br>18 | 17<br>18 | 17<br>19 |          |          |         | 60<br>60    |

in the early cleavage stages since no further cleavage was observed. Although the observations are more difficult in the later stages, careful examination failed to reveal any development subsequent to treatment with azide. If the eggs are removed within 30 minutes after being placed in the azide solution and thoroughly washed they can proceed with their development.

Barnes' (1944) experiments with azide were done at higher temperatures, concentrations, and pH than those described above. Accordingly when the azide ex-

periments were repeated they were done at room temperature (ca. 25° C.), at pH 7.4 and 8.3 (i.e. with and without added hydrochloric acid), and with concentrations up to 0.01 M. The results of these experiments are given in Table III. At both hydrogen ion concentrations, 0.01 M azide caused immediate arrest in all pre-gastrular stages. The 0.005 M concentration used in the early experiments was retested under these conditions and found to give exactly the same results as previously obtained. Controls kept in Ringers buffered at the experimental pH developed normally in all cases, and are not reported in the table.

TABLE III

The effect of azide on development at 25° C.; 1944-5, material from Wisconsin

| Stage at immersion | Conc. (Molar) | pH  | Stage at arrest |
|--------------------|---------------|-----|-----------------|
| 1                  | 0.001         | 7.4 | 9*              |
| 1                  | 0.001         | 8.3 | 9*              |
| 1                  | 0.005         | 7.4 | 1               |
| 1                  | 0.005         | 8.3 | 1               |
| 1                  | 0.01          | 7.4 | 1               |
| 1                  | 0.01          | 7.4 | 1               |
| 1                  | 0.01          | 8.3 | 1               |
| 7                  | 0.005         | 7.4 | 7               |
| 7                  | 0.01          | 7.4 | 7               |
| 9                  | 0.005         | 7.4 | 9               |
| 9                  | 0.005         | 7.4 | 9               |
| 11+                | 0.01          | 8.3 | 12-             |
| 13                 | 0.01          | 8.3 | 13              |

\* There was no delay in reaching this stage.

It is clear that we can offer no support to Barnes' statement that at the concentration and pH she employed, azide, like cyanide, permits eggs to develop to gastrulation.

#### *The effect of NaCN on development at different pH values*

Holtfreter (1943) presented evidence showing that the disruptive effects of strong cyanide solutions (0.1 M to 0.0015 M) can be imitated by potassium hydroxide solutions of equal pH. Although the author did not specifically claim that the oxidation-repressing effects of cyanide are to be regarded as completely irrelevant to its influence on development, it nevertheless seemed advisable to us to clarify the points which were left in an indecisive state by Holtfreter's work. This we did, in our 1944-1945 series of experiments, both by comparing the effects of NaCN solutions brought to pH 7.2 with HCl with those at pH 9.6-9.8, and by determining the effects of solutions of either NaOH or KOH at pH 9.8. The tests were made at about 25°; the cyanide solutions were changed three times daily, the hydroxide solutions once daily.

The results of the NaCN tests completely confirmed our earlier findings (Table IV). The stage in which development was stopped, and the speed with which that stage was reached, was in all cases the same in solutions of equal concentration at both low and high pH. Only after the egg had been in an arrested stage for

more than 12 hours did a difference between the two pH's become evident. At high alkalinity the pigment became streaked, the surface disintegrated, and the egg was in the majority of cases reduced to a loose, fuzzy mass of cells within 36 hours; at low alkalinity the surface was only moderately eroded after 72 hours.

TABLE IV

The effects of NaCN at pH 7.2 and 9.8; 1944-5, material from Wisconsin

| Stage at immersion | Conc. (Molar)  | pH 7.2                   |  | pH 9.6-9.8      |   |
|--------------------|----------------|--------------------------|--|-----------------|---|
|                    |                | Stage at arrest          | Later effects  | Stage at arrest | Later effects   |
| 1<br>1             | 0.003<br>0.006 | Not tested<br>Not tested |  | 7<br>7          | Egg swollen and surface severely depigmented after 36 hrs.                      |
| 7<br>7             | 0.003<br>0.006 | 9<br>9                   | Marked depigmentation after 40 hrs.  | 9<br>9          | Depigmentation after 20 hrs., surface disintegrated after 36 hrs.               |
| 9<br>9             | 0.003<br>0.006 | 11<br>11                 | Blastopore lip disappeared within 20 hrs. after forming                                  | 11<br>11        | Blastopore lip also disappeared. Surface completely disintegrated after 24 hrs. |
| 1                  | 0.004          | 7                        | Surface became mottled but did not disintegrate within 72 hrs.                           | 7               | Complete disintegration within 24 hrs.  |
| 1                  | 0.004          | 7                        | Egg swelled to twice its normal diameter but did not disintegrate within 96 hrs.         | 7               | Complete disintegration within 24 hrs.  |
| 8                  | 0.004          | 9                        | Surface became mottled and egg swelled somewhat, but did not disintegrate within 88 hrs. | 9               | Surface became mottled within 24 hrs., complete disintegration within 38 hrs.   |

The studies with hydroxides revealed that *Rana pipiens* eggs can develop from fertilization to the stage of tail-fin circulation (stage 22, at which they were discarded) at pH 9.8 (i.e.,  $2.5 \times 10^{-4}$  M). Stage 22 was also achieved unevenly if the eggs were immersed in the hydroxide solutions at the stage of the morula (S7), late blastula (S9), mid-gastrula (S11), neurula (S14), muscular movement (S18); in the last two cases the vitelline membrane was removed before the embryos were placed in the alkali solutions. In complete contradiction to Holtfreter's finding that eggs disintegrate in the morula stage in KOH solutions of pH 9.0 to 9.4, we did not observe either retardation or abnormality of development. In three experiments with NaOH and two with KOH, we obtained identical results. Thus we may conclude that the suppressive action of NaCN (or KCN) on living egg is due to the poisonous effect of the CN component.

## DISCUSSION

Both azide and cyanide are effective inhibitors of respiration in the early as well as in the later stages of development (Barnes, 1944; Spiegelman and Steinbach, 1945). The fact that cyanide cannot inhibit at any stage before gastrulation whereas azide can inhibit at all stages, cannot be explained on a respiratory basis. This is even more pointedly demonstrated by the capacity of eggs to develop to gastrulation under anaerobic conditions. The ability of cyanide to depress respiratory rates at all stages clearly proves that it gets into the cells of the early embryos, and consequently a difference in permeability cannot be invoked to explain the difference between the effects of azide and cyanide on development. It is clear from these experiments that, at least in the early stages,  $\text{NaN}_3$  is inhibiting some cyanide-insensitive process necessary for development.

Recent work has served to question conclusions drawn from Keilin's earlier experiments that azide and cyanide are essentially equivalent inhibitors of the Warburg-Keilin system. Stannard (1939) showed that cyanide inhibited the respiration of both resting and active muscle while azide affected active muscle only. Armstrong and Fisher (1940) demonstrated that azide and cyanide behave differently in inhibiting the enzymes controlling the frequency of the embryonic fish heart-beat. Differences in cyanide and azide inhibitions of tissue respiration led Korr (1941) to postulate the existence of different pathways of respiration in resting and stimulated tissues. Ball (1942) suggested different oxidation-reduction potentials for the Atmungsferment-cyanide and Atmungsferment-azide compounds as an explanation of the different effects of the two inhibitors. Winzler (1943), after subjecting the kinetics of the respiratory inhibition by cyanide and azide in yeast to a careful examination, came to the conclusion that cyanide inhibited yeast respiration by three different pathways: (1) by combining with oxidized Atmungsferment; (2) by increasing the apparent  $\text{KO}_2$  of reduced Atmungsferment; and finally (3) by combining with the enzyme which controls the rate-limiting step of the rate of respiration. Azide on the other hand exhibited only one type of inhibition, namely, combination with oxidized Atmungsferment.

Aside from these studies on respiration, others have been made on assimilatory activity of microorganisms. Barker (1936) and Giesberger (1936) showed that suspensions of bacteria could under certain circumstances synthesize carbohydrate from various substrates. Clifton (1937) studied the effect of azide on these syntheses and found them to be completely inhibited. In the presence of azide external substrate was completely oxidized. Clifton and Logan (1939) extended these findings and showed that it was possible to differentially inhibit assimilatory processes with both  $\text{NaN}_3$  and 2, 4-dinitrophenol. Winzler (1940), working with acetate assimilation in yeast, showed that low concentrations of azide, cyanide, or 2, 4-dinitrophenol prevented assimilation. Azide was also shown by Winzler (1944) to prevent the anaerobic assimilation of glucose by yeast without interfering with its fermentation. Winzler, Burk, and du Vigneaud (1944) found that azide in concentrations of  $10^{-4}$  and  $10^{-5}$  molar inhibits completely the anaerobic assimilation of ammonia.

These experiments show that azide, and in certain instances cyanide, can inhibit synthetic processes which are essentially anaerobic in nature and not connected with the Warburg-Keilin system. It seems most probable that it is this

sort of inhibitory activity which is involved in the ability of azide to stop embryonic development. Unfortunately, with the exception of Winzler's (1940) study of acetate assimilation no detailed comparison between the effects of azide and cyanide on synthetic processes has been published. In view of the results reported in the present paper one would venture to predict that such differences will be discovered. It may be noted that one such difference has been found in the case of adaptive enzyme formation in yeast, which is azide sensitive but is not inhibited by cyanide (Spiegelman, 1945). A suggestive finding has been reported recently by Meyerhof (1945), who prepared a solution of adenylypyrophosphatase from yeast by supersonic vibration and found it insensitive to cyanide but highly sensitive to azide. This enzyme, involved as it is in transphosphorylation, might conceivably be a part of the azide sensitive anaerobic synthetic processes.

#### SUMMARY

Previous observations that amphibian eggs can develop up to the beginning of gastrulation in cyanide solutions have been confirmed on eggs of *Rana pipiens*. The effect of cyanide is independent of pH, and eggs can develop into tadpoles in  $2.5 \times 10^{-4}$  molar NaOH or KOH solutions at pH 9.8.

Azide has been found to arrest development immediately in all stages from fertilization to tail-bud formation. The effect is the same from pH 6.6 to pH 8.3.

These differences are discussed in the light of recent studies on the effects of azide and cyanide on respiratory, assimilatory, and phosphorylative processes.

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# THE BIOLOGICAL BULLETIN

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## STUDIES ON THE BIOCHEMISTRY OF TETRAHYMENA. IV. AMINO ACIDS AND THEIR RELATION TO THE BIOSYNTHESIS OF THIAMINE

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It was reported earlier (Kidder and Dewey, 1942) that two species of *Tetrahymena* were able to carry out the synthesis of thiamine, if provided with a substance found mainly in the leaves of plants. This substance was called Factor S and was found in highest concentration in alfalfa leaf meal but could not be demonstrated from materials of animal origin. Factor S was characterized by its solubility in water and alcohol (up to 75 per cent), insolubility in ether and acetone, stability to prolonged heat in the presence of either alkali or acid, and its stability to ultraviolet radiation. It was shown to be dialyzable through cellophane and not to be precipitated by the salts of heavy metals. It was shown that *Tetrahymena* gave optimal growth in a medium consisting of "vitamin-free" casein, salts and a heat- and alkali-treated water extract of alfalfa meal. Very little growth occurred in the absence of the alfalfa extract and the addition of thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, pimelic acid, *i*-inositol, uracil, or *p*-aminobenzoic acid either singly or in combination had no significant effect. Inasmuch as the heat- and alkali-treated alfalfa extract was certainly free of thiamine it was concluded that *Tetrahymena* could synthesize the thiamine required for its metabolic needs when supplied with Factor S. It was suggested that Factor S possibly acted as a catalyst necessary for the synthesis of the thiamine molecule.

It was recognized that the alfalfa extract used contained Factors I and II (Dewey, 1941; 1944) and we now know that the casein base contained Factor III (Kidder and Dewey, 1945a).

This work was criticized by Hall and Cosgrove (1944) on the basis that the "vitamin-free" casein used for the base medium was not free of thiamine. They reported growth of their strain of *Tetrahymena* in heat- and alkali-treated casein in the presence of thiamine and not in its absence. This criticism was shown to be invalid (Kidder and Dewey, 1944) when an extension of the earlier studies was carried out, using heat- and alkali-treated base media (casein, casein hydrolysate, gelatin, gelatin hydrolysate). It was then found that heat and alkali treatment of

<sup>1</sup> Aided by grants from the Morgan Edwards Fellowship Fund, the Manufacturers Research Fund for Bacteriology and Protozoology of Brown University.

whole casein produced toxic substances which could not be overcome by thiamine addition for *T. geleii* W but could to a slight extent for *T. geleii* H. In tryptophane-supplemented gelatin (Harris), however, indefinitely transplantable growth was possible after all of the thiamine had been destroyed. The addition of thiamine did not affect the generation time but did increase significantly the maximum yield and survival. The addition of heat- and alkali-treated alfalfa extract decreased the generation time and raised the maximum yield to optimal for the species, and the addition of thiamine had no significant effect. This was interpreted as meaning that gelatin possessed low concentrations of Factors I, II, and S and that the final cessation of growth was due to the depletion principally of Factor S, as the addition of thiamine did raise the maximum yield.

One of the difficulties encountered in the earlier work was the separation of Factor S from Factors I and II. The heat and alkali treatment of peptones seemed to destroy the Factor I activity, but toxic substances were produced which rendered the medium inferior for our tests. Nevertheless, it was possible to show that lead acetate precipitate (containing no factor S) from plant material could replace the heat- and alkali-destroyed fraction only if thiamine was added. This was taken to mean that peptone contained no Factor S but did contain Factor II which was stable to the treatment used for dethiaminization, and Factor I which was unstable. It was recognized that little more could be done until active preparations of Factors I and II could be obtained which were essentially free of both Factor S and toxic materials.

Recently we have been able to obtain such a preparation and it has been possible to test the activity of Factor S. This work, to be reported here, while confirming our earlier conclusions on thiamine synthesis, has forced us to alter our original theory concerning the role of Factor S in the metabolic activities of *Tetrahymena*.

#### MATERIAL AND METHODS

The organism used in the present study was the ciliated protozoan *Tetrahymena geleii* W, which is the strain used in the previous studies on thiamine synthesis (Kidder and Dewey, 1942; 1944). All work was done with pure (bacteria-free) cultures. The ciliates were grown in 2 ml. quantities of media in Pyrex tubes according to the technique described elsewhere (Kidder and Dewey, 1945b). All media, made with water twice distilled over permanganate in an all-Pyrex still, were adjusted to give a final pH of 6.8–7.0 and sterilization was by autoclaving. Serial transplants were made and results are recorded only after the third transplant. Transplants were made at 72 hour intervals using a bacteriological loop delivering approximately 0.008 ml. of fluid. Incubation was at 25° C. Population densities were determined by the direct counting technique (Kidder, 1941). All glassware used in this investigation was made chemically clean with sulfuric-dichromate solution, thoroughly rinsed and air dried before use.

In order to eliminate the possibility of cotton fibers contributing substances to the medium, Pyrex wool plugs were used extensively. It was found helpful to flame the protruding ends of the plugs until a thin crust had formed to eliminate the annoying strands inevitably present in this type of plug. This treatment fuses enough of the Pyrex strands to cause the plugs to hold their shape and increases appreciably the ease with which they may be handled.

Two types of base media were used for most of this work. One was 0.5 per cent hydrolyzed Eastman purified calfskin gelatin (Lot no. 144). This hydrolysate was prepared by refluxing 100 gr. of gelatin in one liter of 25 per cent  $\text{H}_2\text{SO}_4$  for 5 hours, removing the sulfate as  $\text{BaSO}_4$  and reducing to the required concentration. Hydrolysate prepared with  $\text{HCl}$  was also used and the two were similar in every way. The gelatin hydrolysate was supplemented in all cases with 0.01 per cent *l*(-)-tryptophane and (with one exception to be noted later) with 0.02 per cent *dl*-valine. This base medium will be referred to as EGH.

The second type of base medium employed was a mixture of the eleven amino acids found to give optimum growth for this strain of *Tetrahymena geleii* (Kidder and Dewey, 1945a). These amino acids with the concentration in mg. per cent of each were as follows: *l*(+)-arginine monohydrochloride—82; *l*(-)-histidine monohydrochloride—10; *dl*-isoleucine—35; *dl*-leucine—35; *dl*-lysine—60; *dl*-methionine—34; *dl*-phenylalanine—14; *dl*-serine—4; *dl*-threonine—20; *l*(-)-tryptophane—10; *dl*-valine—20. This base medium will be referred to as 11 AA. The sources of the amino acids used have been given elsewhere (Kidder and Dewey, 1945b).

Inasmuch as our primary concern was with thiamine all media were made up to contain other known growth factors, minerals and sugar to insure against limiting factors outside the scope of this investigation. Accordingly to our base media the following were always added:

|   | mg./ml.        |
|---|----------------|
| Difco bacto dextrose.....                       | 2.00           |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ..... | 0.10           |
| $\text{K}_2\text{HPO}_4$ .....                  | 0.10           |
| $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ..... | 0.05           |
| $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ..... | 0.00125        |
| $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ..... | 0.00005        |
| $\text{ZnCl}_2$ .....                           | 0.00005        |
|   | Micrograms/ml. |
| Biotin methyl ester.....                        | 0.00005        |
| Calcium pantothenate.....                       | 0.10           |
| Nicotinamide.....                               | 0.10           |
| <i>i</i> -Inositol.....                         | 1.00           |
| Choline chloride.....                           | 1.00           |
| <i>p</i> -Aminobenzoic acid.....                | 0.10           |
| Pyridoxine hydrochloride.....                   | 0.10           |
| Uracil.....                                     | 0.10           |
| Folic acid <sup>2</sup> .....                   | 0.01           |
| Riboflavin.....                                 | 0.10           |

The sources of the salts and growth factors have been given earlier (Kidder and Dewey, 1945b).

Our preparation containing Factors I, II, and III was made from Liver Fraction L.<sup>3</sup> Fifteen grams of Liver Fraction L was dissolved in 750 ml. of distilled water, adjusted to pH 4.5, and extracted continuously for 96 hours in a liquid-liquid extracting apparatus (Wilson, Grauer, and Saier, 1940) with 750 ml. of *n*-butyl alcohol. The extracted material was freed of butyl alcohol, neutralized and

<sup>2</sup> Folic acid concentrate with a "potency" of 5000, furnished through the courtesy of Dr. R. J. Williams.

<sup>3</sup> Furnished through the courtesy of Dr. David Klein and the Wilson Laboratories.

the volume reduced to 300 ml. This was designated 12L, and was found to contain adequate amounts of Factors I, II, and III. The pH of this preparation was adjusted to 9.5–10.5 with NaOH and heated in the autoclave at 123° C. for one hour for dethiaminization. This preparation will be designated 12L1, which was found to be free of Factor S activity. 12L1 was used as a supplement in a final concentration of 1:20.

Preparations containing Factor S were obtained from alfalfa meal. Water extract of alfalfa, as previously described (Kidder and Dewey, 1942; 1944), was heated in the autoclave at 123° C. for one hour at pH 9.5–10.5 to insure the destruction of thiamine. This preparation, designated A, was used in a final concentration of 1:10.

### RESULTS

When Liver Fraction L is heated with alkali to destroy thiamine, changes take place which make it inferior as a source of supplementary factors for *Tetrahymena*. The addition of thiamine does not completely overcome these toxic effects, although the inhibition is less than that produced when proteose-peptone is dethiaminized. It was found, however, that toxic materials were not produced upon heating provided the Liver Fraction L was extracted previously with butanol. The butanol extraction was used originally for the removal of pyridoxin and riboflavin (to be reported in detail later).

TABLE I

Growth in EGH and 11 AA with and without added Factor S from dethiaminized alfalfa extract (A) and with and without added thiamine. All tubes contain 12L1 and the numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

| Base | Additions |          |         |              |
|------|-----------|----------|---------|--------------|
|      | 0         | Thiamine | A       | A + Thiamine |
| EGH  | 3,100     | 305,000  | 75,000  | 290,000      |
| 11AA | 120,000   | 310,000  | 165,000 | 300,000      |

It was found that optimum growth resulted when a gelatin hydrolysate medium (with tryptophane, valine, and ten known growth factors), referred to as EGH, was supplied with 12L1 and thiamine, and very low growth occurred when the thiamine was omitted. The 12L1 was low in Factor S yet contained adequate amounts of Factors I, II, and III. This offered the opportunity to test the mode of action of Factor S, which could now be supplied from plant material without reference to the amounts of essential growth factors. Accordingly tests were set up using both EGH and 11 AA as base media, both supplemented with 12L1. To these base media were added various combinations of dethiaminized alfalfa extract (A) and thiamine. The results which were expected, namely the failure of growth unless either thiamine or Factor S was present, were not realized in 11 AA. Table I shows that very little growth occurred in the media based on EGH unless thiamine or Factor S was supplied but relatively good growth was obtained in the amino acid mixture in the absence of both. It will also be noted that thiamine is much more stimulatory, under these conditions, than is Factor S.

It was apparent from the foregoing results that the ability of *Tetrahymena* to synthesize thiamine was not dependent on the presence of Factor S when 11 AA was used as the base medium. This led to the conclusion that either some amino acid or combination of amino acids in the gelatin hydrolysate was blocking the synthetic mechanisms or that materials in the 12L1 were causing the block, the latter block being removed by some combination of the pure amino acids not present in the gelatin hydrolysate. The first of these possibilities was tested by making up an amino acid mixture based exactly on the published analysis for gelatin, but adding both tryptophane and valine (indispensable for this species). The ciliates behaved in this synthetic gelatin hydrolysate just as they had in 11 AA, so it was apparent that the first of the possibilities was untenable. The only known difference between the synthetic gelatin hydrolysate and EGH from a qualitative point of view was the inclusion in the former mixture of synthetic unnatural isomers (in the *dl* form, because of availability) of a number of the amino acids.

The addition of 11 AA to EGH plus 12L1 resulted in good growth without the addition of either thiamine or Factor S. This led us to test the effect of omitting each of the 11 amino acids singly from the 11 AA added to EGH. These results were inconclusive as fair growth occurred in all tubes. This was taken to mean that more than one of the 11 amino acids could counteract the inhibition to thiamine synthesis.

TABLE II

Growth of EGH with the addition of varying concentrations of racemic mixtures of amino acids. All tubes contain 12L1. The numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

| Amino acid                  | Concentration of amino acid added (mg./ml.) |        |        |         |         | Control,<br>nothing added |
|-----------------------------|---|--------|--------|---------|---------|---------------------------|
|                             | 0.1   | 0.3    | 0.5    | 0.8     | 1.0     |                           |
| dl-phenylalanine            | 84,000                                      | 80,000 | 82,000 | 94,000  | 101,000 | 3,800                     |
| dl-methionine               | 92,000                                      | 8,600  | 8,000  | 6,000   | 7,800   |                           |
| dl-serine                   | 58,000                                      | 82,000 | 97,000 | 114,000 | 110,000 |                           |
| dl-norleucine               | 31,000                                      | 11,500 | 0      | 0       | 0       |                           |
| dl-aspartic acid            | 21,000                                      | 46,000 | 51,000 | 87,000  | 62,000  |                           |
| dl-isoleucine               | 11,500                                      | 56,000 | 70,000 | 97,500  | 60,000  |                           |
| dl-lysine monohydrochloride | 4,500                                       | 15,000 | 58,000 | 61,000  | 78,000  |                           |
| dl-threonine                | 6,000                                       | 31,000 | 42,000 | 66,000  | 81,000  |                           |
| dl-homcystine               | 10,500                                      | 33,000 | 11,000 | 8,000   | 6,400   |                           |
| dl-alanine                  | 3,000                                       | 4,200  | 26,000 | 11,000  | 4,500   |                           |
| dl-glutamic acid            | 6,500                                       | 7,500  | 12,500 | 21,000  | 37,000  |                           |

The next set of experiments was designed to determine whether or not the addition of single amino acids to the gelatin hydrolysate medium could counteract the inhibition to thiamine synthesis. Arbitrary amounts of each of nineteen amino acids were added to EGH. Thiamine synthesis occurred to a marked degree in some of the tubes, moderately in others and very little in some. In all cases where the inhibition was not removed the amino acid used was in its natural form while those amino acids which were most effective were synthetic.

This set of experiments was repeated using varying concentrations of the synthetic amino acids and some of the results are given in Table II. It will be seen that the effectiveness of the amino acids in releasing the inhibition of thiamine synthesis varied with the amino acid and the concentration. Phenylalanine was the most effective throughout the range of concentrations used while methionine was most effective in the lowest concentration. Norleucine was moderately effective at a concentration of 0.1 mg. per ml. but was toxic at 0.5 mg. per ml. or higher. These results indicated that the unnatural isomers were in some way able to release the inhibition of thiamine synthesis. It seemed more probable that the ratio between the two isomers was not the explanation, as some release of inhibition was found with some of the nonsynthetic amino acids. It is known that in the preparation of amino acids from natural sources some racemization is likely to occur and this might account for the small amount of activity.

TABLE III

Comparison of the effect of the natural isomer (1+) and the unnatural isomer (1-) of isoleucine, added to EGH + 12L1. Numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

| Amino acid      | Concentration of amino acid added (mg./ml.) |        |        |        |        |                           |
|-----------------|---|--------|--------|--------|--------|---------------------------|
|                 | 0.1   | 0.3    | 0.5    | 0.8    | 1.0    | Control,<br>nothing added |
| 1(+)-isoleucine | 7,800                                       | 4,100  | 6,300  | 9,800  | 11,500 | 3,100                     |
| 1(-)-isoleucine | 42,000                                      | 91,000 | 81,000 | 68,000 | 21,000 |                           |

This was shown to be the probable explanation by two sets of experiments. We had samples of natural *l*(+)-isoleucine, unnatural *l*(-)-isoleucine and synthetic *dl*-isoleucine. A comparison of the figures for *dl*-isoleucine in Table II with those in Table III shows that *l*(-)-isoleucine is effective in approximately one half the required concentration of *dl*-isoleucine. This is what is to be expected if only the

TABLE IV

Comparison of the effect of the natural isomer (1-) and the racemic mixture (*dl*) leucine, added to EGH + 12L1. Numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

|              | Concentration of amino acid added (mg./ml.) |       |        |        |        |                           |
|--------------|---|-------|--------|--------|--------|---------------------------|
|              | 0.1   | 0.3   | 0.5    | 0.8    | 1.0    | Control,<br>nothing added |
| l(-)-leucine | 2,500                                       | 3,500 | 10,500 | 14,000 | 13,500 | 3,800                     |
| dl-leucine   | 4,100                                       | 5,500 | 26,000 | 29,000 | 31,000 |                           |

unnatural isomer is effective in the removal of thiamine synthesis inhibition. The effectiveness of *l*(+)-isoleucine is low and increases with the concentration. This could be due to the occurrence of some racemization during its preparation.

When natural leucine was compared to *dl*-leucine the former was found to be less effective in the release of the synthesis inhibition (Table IV). The difference

here, however, was not as marked, as the natural form appears to contain a considerable quantity of racemic mixture and the synthetic leucine is rather low in activity. It should be noted that we used Kahlbaum *dl*-leucine as this was found previously (Kidder and Dewey, 1945b) to be free of isoleucine, a common contaminant of many brands of synthetic leucine (Hegsted and Wardwell, 1944).

Inasmuch as EGH contained added *dl*-valine it was thought advisable to determine whether the unnatural isomer of this amino acid might be responsible for the ability of the ciliates to grow at all without added thiamine, Factor S or unnatural isomers of amino acids (see controls in Tables I-IV). Accordingly EGH minus valine was tested with varying concentrations of *dl*-valine with and without thiamine. Table V shows that without thiamine, very little growth occurs with no

TABLE V

Effect of the addition of *dl*-valine to EGH (minus valine). All tubes contain 12L1. Numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

|                | Concentration of <i>dl</i> -valine (mg./ml.) |         |         |         |         |         |         |
|----------------|--|---------|---------|---------|---------|---------|---------|
|                | 0  | 0.05    | 0.1     | 0.3     | 0.5     | 0.8     | 1.0     |
| Minus thiamine | 150  | 2,500   | 4,000   | 7,500   | 16,000  | 37,000  | 45,000  |
| Plus thiamine  | 190,000                                      | 210,000 | 265,000 | 310,000 | 305,000 | 325,000 | 315,000 |

added valine, and that the inhibition to thiamine synthesis is counteracted more effectively the higher the concentration of added *dl*-valine. With added thiamine, however, the addition of valine had little effect. This indicates that the sample of gelatin used differs from our previous sample of Eastman de-ashed gelatin in that it contains nearly optimum amounts of natural valine for this species. It had previously been found (Kidder and Dewey, 1945b) that Eastman de-ashed gelatin would not support growth of *Tetrahymena geleii* W without added valine, even in the presence of thiamine. The fact that transplantable, though very low, growth occurs without the addition of any unnatural isomers of amino acids may mean that the inhibition to thiamine synthesis is never complete or that some racemization of the amino acids has occurred during hydrolysis.

When thiamine was added (0.1 micrograms per ml.) to any of the above described combinations, growth was always raised to approximately 300,000 ciliates per ml. Thiamine, therefore, although it can be synthesized by the ciliates, is very active as a stimulatory substance. It was of interest and importance to determine the amount of stimulation produced by different concentrations of thiamine when added to EGH plus 12L1; EGH plus 12L1 and one of the active amino acids; EGH plus 12L1 and Factor S; and 11 AA plus 12L1. Figures 1-4 show a summary of the activity of various concentrations of thiamine. The lowest concentration tested was 0.005 millimicrograms per ml. and in every case this amount gave significant stimulation. The stimulation was roughly proportionate to the concentration up to 0.001 micrograms per ml. In all cases, after this point, the amount of growth was increased more gradually but reached approximately the 300,000 level at 0.01 micrograms per ml. of thiamine when inhibition to thiamine synthesis was absent or removed. Ten times this amount of thiamine was required to raise

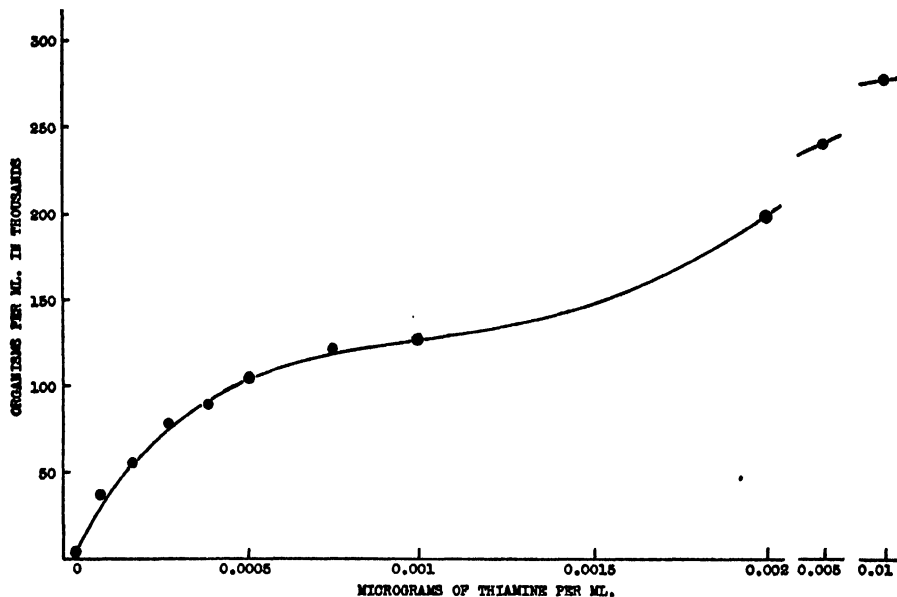


FIGURE 1. Curve of population densities at various concentrations of thiamine hydrochloride with gelatin hydrolysate (EGH) and dethiaminized butanol extracted Liver Fraction L (12L1) as base. The concentration of organisms was determined from the third transplant after 72 hrs. of growth.

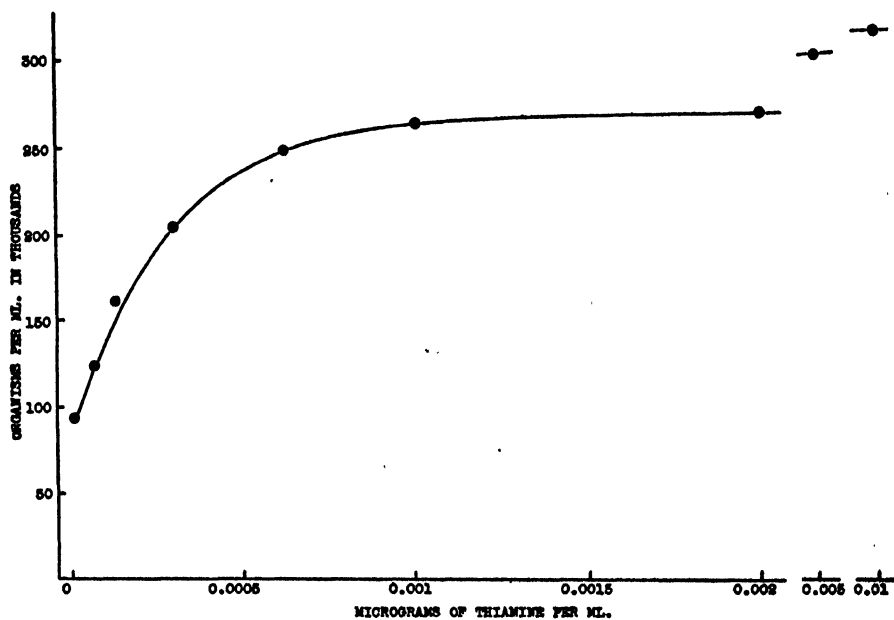


FIGURE 2. Curve of population densities at various concentrations of thiamine hydrochloride with EGH, 12L1 and *dl*-serine (0.5 mg./ml.) as base. Third transplant determinations after 72 hrs. of growth.

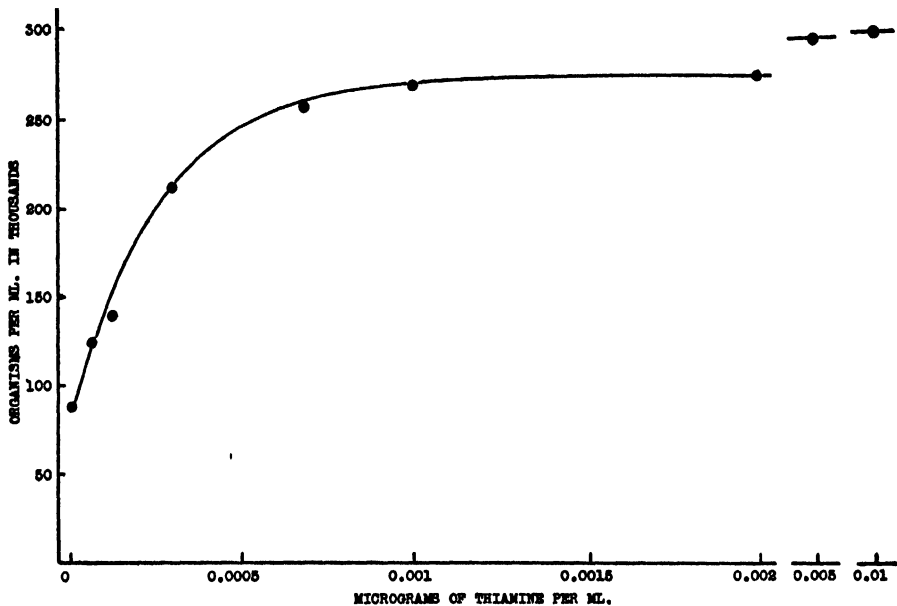


FIGURE 3. Curve of population densities at various concentrations of thiamine hydrochloride with EGH, 12L1 and dethiaminized alfalfa extract (A) as base. Third transplant determinations after 72 hrs. of growth.

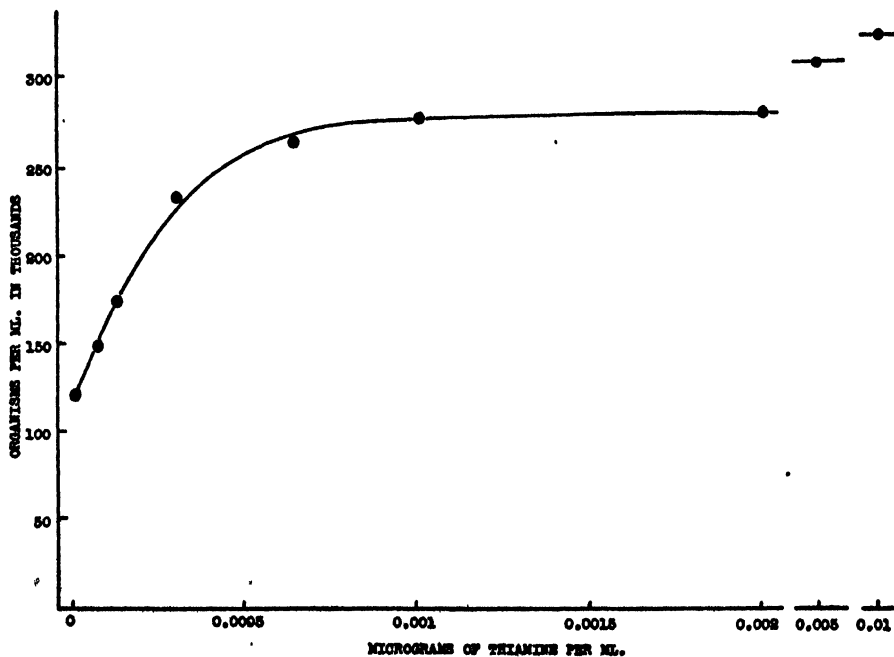


FIGURE 4. Curve of population densities at various concentrations of thiamine hydrochloride with the amino acid mixture (11 AA) and 12L1 as base. Third transplant determinations after 72 hrs. of growth.

the population to 300,000 per ml. where inhibition was pronounced (Fig. 1). These results show that *Tetrahymena* is far more sensitive to thiamine below a concentration of 0.001 micrograms per ml. than to higher concentrations.

An interesting and perhaps important point to be noted in the data shown in Figure 1 is the inflection which occurs in the curve above the 0.001 microgram per ml. level. The reasons for this inflection are not clear, although it seems possible that thiamine may be performing a double role where inhibition is pronounced. It may be supplying the vitamin needs of the organisms at the lower levels and acting to remove other inhibitions to growth as the concentrations increase.

Only the intact molecule of thiamine is capable of giving optimum stimulation. When the pyrimidine portion of thiamine (2-methyl-5-ethoxymethyl-6-amino pyrimidine) <sup>4</sup> or the thiazole portion (4-methyl-5-beta-hydroxyethyl thiazole) <sup>4</sup> were added separately or together some release of inhibition occurred. Table VI shows

TABLE VI

Growth in EGH plus 12L1 with varying concentrations of the thiazole and pyrimidine components of the thiamine molecule. Numbers represent organisms per ml. in the third serial transplant after 72 hrs. of growth.

|  | Concentration (micrograms/ml.) |        |        |       |       | Control,<br>nothing added |
|--|--------------------------------|--------|--------|-------|-------|---------------------------|
|  | 0.001                          | 0.005  | 0.01   | 0.1   | 0.5   |                           |
| Thiazole                                 | 3,000                          | 8,400  | 20,000 | 1,400 | 1,000 | 2,900                     |
| Pyrimidine                               | 1,200                          | 5,300  | 17,500 | 3,200 | 2,600 |                           |
| Thiazole and pyrimidine<br>(total conc.) | 2,500                          | 11,000 | 24,000 | 2,400 | 2,500 |                           |

the results of these experiments. Both thiazole and pyrimidine produce stimulation in low concentrations but are mildly toxic at concentrations of 0.1 micrograms per ml. or higher. Thiazole and pyrimidine behave much the same as Factor S or the unnatural isomers of the amino acids, although to a less degree. They appear to cause the release of the thiamine synthesis inhibition and are themselves inhibitory in high concentrations.

#### DISCUSSION

It appears from the foregoing results that there are substances present in natural materials which can block the synthetic mechanisms of *Tetrahymena*. Under the conditions of our experiments this blocking occurred specifically in the mechanism or mechanisms for the synthesis of the thiamine molecule. That this ciliate can synthesize thiamine, as was pointed out earlier (Kidder and Dewey, 1942; 1944) cannot be doubted, when the blocking substance is absent or the block is released. In our earlier work (Kidder and Dewey, 1942; 1944) where dethiaminized alfalfa extract was used as the supply of Factors I and II (Factor III was present in the casein and gelatin preparations; Kidder and Dewey, 1945a), it might be questioned whether the growth obtained in the absence of thiamine might be the result of no inhibitory substance rather than the presence of Factor S. However, it must be re-

<sup>4</sup> Both the thiazole and the pyrimidine used were furnished through the courtesy of Dr. George W. Lewis and Merck and Co.

membered that the addition of alfalfa extract to EGH plus 12L1 (which contains the inhibitory substance) removed the block. Whatever Factor S is, it is able to release the block to thiamine synthesis. But it is also seen that the unnatural isomers of the amino acids can act in a similar manner, so this reaction is far from specific as to counteracting substances. It was formerly proposed (Kidder and Dewey, 1942) that Factor S might act as a catalyst to the reaction wherein the thiamine molecule was synthesized. This hypothesis appears to be no longer tenable.

It does not seem likely that Factor S is, in reality, nothing more than racemic amino acids, for two reasons. If enough racemization occurred during the heat treatment of the alfalfa extract to account for the activity found then the same amount of racemization should have taken place in the heat treatment of 12L to produce 12L1. It was found, moreover, upon assaying the alfalfa extract for the indispensable amino acids for *Tetrahymena* that it did not contain enough of any one of the ten to support growth, when used in the concentration employed here. But a similar assay of 12L1 demonstrated almost optimum amounts of lysine; approximately half optimal amounts of arginine, threonine, and valine; and traces of histidine, isoleucine, leucine, and phenylalanine. It seems at present that Factor S represents some material present in alfalfa and the leaves of other plants (Kidder and Dewey, 1942), the activity of which is shared by the unnatural isomers of many of the amino acids.

The relation of amino acids to the ability of organisms to synthesize vitamins has been pointed out before. Snell and Guirard (1943) showed that alanine could replace pyridoxine for *Streptococcus fecalis* R (*S. lactis* R) and that alanine functioned to counteract the toxicity of glycine. It does seem strange, however, that the unnatural isomers appear to function in the release of thiamine synthesis inhibition for *Tetrahymena*. In nature this organism, being largely a bacteria feeder, probably would never be called upon to use its thiamine synthesis mechanism. The use of its ability to synthesize thiamine, therefore, is admittedly the result of artificial environmental conditions, as is also the very contact with the unnatural isomers of the amino acids.

It is apparent that, although *Tetrahymena* does possess the ability to synthesize thiamine, this vitamin is a potent stimulant to reproduction, size (Kidder and Dewey, 1944), and longevity (Johnson and Baker, 1943). Thiamine must, therefore, be included in complete media for this ciliate, but the amount needed appears to be less than has been previously used (Hall and Cosgrove, 1944; Kidder and Dewey, 1942; 1944).

It has been stated previously (Lwoff and Lwoff, 1938; Kidder and Dewey, 1942; 1944; Hall and Cosgrove, 1944) that heating peptones or proteins with alkali renders the media inferior for the growth of *Tetrahymena*. This condition could be partially counteracted for some strains by the addition of thiamine. The explanation appears now to rest in the partial destruction of serine, for we have found that if 11-AA is heat- and alkali-treated growth (with added 12L1) is very low but returns to normal with the addition of serine. Increased growth results with the addition of thiamine alone, however, indicating that this vitamin can replace serine. Or that serine (a dispensable but highly stimulatory amino acid in the presence of thiamine; Kidder and Dewey, 1945b), is one of the necessary factors for the synthesis of vitamin B<sub>1</sub>.

The relationship which exists between the concentration of thiamine and the concentration of ciliates (Figures 1-4) might suggest that this organism would be useful for assay purposes. It would be difficult, however, to assay natural products for thiamine in a base medium composed of EGH plus 12L1, the only combination which gives a low blank, because of the likelihood of the introduction of Factor S or other materials of like nature with the substance to be assayed. Although we have not attempted to do this, it might be possible to arrange conditions so that 11 AA (Fig. 4) could be used and the values calculated as differences. Experiments directed to this end might prove valuable as the present microbiological methods are not entirely satisfactory. The majority of organisms used are stimulated by the thiamine components as well as by the whole molecule (Sarett and Cheldelin, 1944), require complex base media (Williams, 1942), or require many days of growth before results can be obtained (Robbins and Kavanagh, 1937).

#### SUMMARY

1. In Eastman gelatin hydrolysate (EGH) and Factors I, II, and III from Liver Fraction L (heat- and alkali-treated to destroy thiamine) the ciliate *Tetrahymena geleii* W grows very poorly without added thiamine.

2. A mixture of amino acids (11 AA) with the dethiaminized liver fraction supports fair growth without added thiamine.

3. There appear to be substances in the liver fraction or the gelatin hydrolysate or both which specifically block the mechanism for the biosynthesis of thiamine.

4. This block can be released by Factor S from alfalfa extract or by the unnatural isomers of a number of amino acids.

5. Some release of the inhibition to thiamine synthesis is produced by a few of the natural amino acids but this is probably due to the presence of low concentrations of unnatural isomers which result from racemization during preparation.

6. The unnatural isomer of isoleucine (the only unnatural isomer available for testing) was found to be active in approximately one half the concentration of the *dl*-isoleucine.

7. Thiamine is extremely stimulatory in low concentrations.

8. The thiazole and pyrimidine components are slightly stimulatory but this stimulation appears to be due to their ability to cause some release of the thiamine synthesis inhibition.

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# CERTAIN CHEMICAL FACTORS INFLUENCING ARTIFICIAL ACTIVATION OF NEREIS EGGS <sup>1, 2</sup>

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## INTRODUCTION

Stimulation must involve physicochemical changes within cells, and the nature of such changes has been the subject of much investigation, both experimental and speculative. The process of fertilization, and the closely related process of activation in artificial parthenogenesis, have attracted special attention; and evidence has been presented for a number of interesting interpretations of this type of activation. This report concerns a group of experiments indicating a peculiar relation of picric acid to the artificial activation of the eggs of *Nereis*. The proper interpretation of these experiments might contribute to the understanding of the stimulatory process. The experiments described developed from incidental observations in connection with heat-activation, during investigations concerned with the more general question of the mode of action of heat on protoplasm.

The peculiarities of heat-activation of the unfertilized *Nereis* egg were first described by Just (1915), who was able to interpret all his data in harmony with Lillie's "fertilizin" theories. In particular, Just attributed the gradual loss of sensitivity to heat, in eggs left standing in sea water, to the diffusion from them of some fertilizin-like substance, essential to the activating process. Heilbrunn (1925) took exception to this notion, in suggesting a "colloid chemical" interpretation of heat-parthenogenesis; he believed the decrease in sensitivity to heat might be due to the gradual loss of CO<sub>2</sub> from the medium, resulting in alkalization of the intracellular fluid. Heilbrunn described three experiments in which the addition of 2-4 volumes per cent of n/10 HCl to old insensitive egg-suspensions restored their original sensitivity to heat.

To reveal a possible general relation between intracellular acidity or carbon dioxide concentration and the response of cells to increased temperatures, these three observations were extended. Heilbrunn's findings were in part confirmed; but with the accumulation of large numbers of experiments, considerable variation was encountered in the response of the heat-sensitivity of the eggs to increased CO<sub>2</sub> concentration through acidification of the sea water. Though such pronounced effects as described by Heilbrunn were often repeatable, as many batches of eggs seemed totally unresponsive to the same treatment. In the course of testing several organic acids in this connection, however, the anomalous properties of picric acid (2, 4, 6-trinitrophenol) came to light. Extension of these properties to processes of activation by means other than heat was then attempted.

<sup>1</sup> This study was carried out under the direction of Dr. L. V. Heilbrunn. I gratefully acknowledge his helpful suggestions throughout the investigations, and his valuable assistance in interpreting the results.

<sup>2</sup> A dissertation submitted to the faculty of the Department of Zoölogy of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

## MATERIALS AND METHODS

Ripe females of the heteronereis form of *Nereis limbata* were captured between 8 and 10 p.m., as described by Lillie and Just (1913), and kept singly or in pairs in about 200 ml. of sea water in finger bowls overnight. These were kept cool either on a salt water bench in a stream of sea water, in a refrigerator, or in a room maintained at 15° C. The last was found by far the most satisfactory in maintaining the worms with eggs intact, with apparently no ill effects. A few worms shed their eggs during the night even at this reduced temperature; these were discarded.

All experiments were begun by the transfer of one or two *Nereis* to a Stender dish containing 25 ml. of sea water. The animals were cut transversely to release the eggs, and the carcasses were quickly removed. The eggs were then concentrated toward the center of the dish by gentle rotation. Either a small quantity of an especially dense suspension of eggs was then removed to another dish, or nearly all of the supernatant fluid was withdrawn by suction, and replaced by fresh sea water. All eggs were washed in this manner through at least another change of sea water, before use. In the earliest work, samples were always tested for normalcy by treatment with sperm from males caught in the same swarm. Only very rarely was any egg ever found which did not become normally fertilized, and all samples showed well over 99 per cent germinal vesicle breakdown. Since this dependability of *Nereis* eggs is well known, and since all danger of accidental contamination of eggs with sperm was to be avoided, no such tests were made in most of the later work. Experiments were always begun on the day following capture, so that the lapse of time between capture and the first treatment was never more than 20 hours, and was only rarely over 15 hours.

All transfers of eggs were made with ordinary medicine droppers. All treatments and exposures, unless otherwise indicated, were made in a volume of 25 ml.; the egg-suspensions were of such a density that, upon settling of the eggs to the bottom, no more than half, and usually much less, of the bottom of the container was covered with a single layer. Stender dishes of about 35 ml. capacity were used except for the exposures to high temperatures; the latter were carried out in 50 ml. beakers, in which the thermal insulation is much reduced. The beakers were immersed in a small deKhotinsky constant-temperature bath to a depth 2-3 mm. above the surface of the inside liquid. The temperature of the fluid within the beakers was brought to equilibrium (at slightly less than half a degree lower than the bath temperature) before the addition of 0.3-0.5 ml. of the egg-suspension. The activating temperature used varied between 33° and 35° C., as in Just's work (1915), but was held constant to within 0.1 of a degree for any single series of tests.

Since it soon became evident that the degree of stirring had a considerable effect on the response to heat, a standard policy in this regard was always followed: upon deposition of the eggs in the warm beakers, the pipette was filled and emptied ten times successively within 4-5 seconds. This was repeated 4 minutes after the beginning of the exposure; and the beaker was removed after 5 minutes of exposure, at which time a sample of 5-8 ml. was removed to a Syracuse watch glass. In some of the earlier work, the second stirring was performed at 15 minutes, the beakers removed at 20 minutes. This exposure, which is approximately Just's optimum, yielded a better percentage of swimmers, but the shorter exposure was found to

produce the maximal amount of germinal vesicle breakdown, and was much more convenient in extended series of tests. A few tests indicated that further stirring and longer exposures led to no increase in the percentage of activation. A further trial showed that the immediate removal to Syracuse dishes was not essential; when the beakers were allowed to cool of their own accord, the residual heat did not affect the percentage of activation.

For counts of activation, 5–8 ml. of each egg-suspension were examined in a Syracuse watch glass at a magnification of about 100  $\times$ . In certain cases involving a doubtful response, compression of the eggs between a slide and coverslip, as suggested by Heilbrunn and Wilbur (1937), and a higher magnification were necessary. The counts were made on the basis of the breakdown of the germinal vesicle, a reaction which normally occurs soon after fertilization. Counts were begun at a minimum of 2 hours after the application of the treatment in question. The advantages of the nuclear criterion are its rapidity of onset, its definite character (ordinarily admitting of easy and certain classification in counting), and its ready susceptibility to quantitative expression; the criterion is well established in work on artificial activation of this form. However, the fact should not be overlooked that the mere breakdown of the germinal vesicle in response to stimulation is seldom followed by development even approaching the normal, and there is rarely any cleavage at all. Various types of monsters are produced, mostly of the type described as due to "differentiation without cell-division," common in annelids. All of the types of stimulation used were capable of producing at least a small percentage of swimming forms, though seldom was anything like a normal trochophore seen. All counts were of 100 or 200 eggs selected by random movement of the watch glass on the stage of the microscope.

## RESULTS

Upon standing in sea water, almost all batches of eggs showed a gradual loss of sensitivity to heat, as described by Just (1915); a few, however, showed a very definite increase in sensitivity, after washing and long standing. This might perhaps be attributable to the washing away of inhibitors in the body fluids (Just, 1915); but the most pronounced of these exceptions was in a special batch in which the eggs stood in a deep layer at the bottom of a narrow container. Thus the responsible factor may have been the high  $\text{CO}_2$  tension, in accordance with Heilbrunn's views (1925). Of several organic acids tested, however, only picric acid produced a consistent and pronounced reversal of this loss of sensitivity to heat. After a batch of eggs had become nearly or quite heat-insensitive, a bath of 15 minutes or more in sea water to which picric acid had been added to a concentration of about M/1000 (pH 6.6) was sufficient to elicit a significant response to the subsequent heat treatment in sea water. Yet the presence of the acid in the heat-treated suspensions completely prevented the activation of the eggs; if a response was to be obtained, the eggs had to be transferred back to sea water for the heat treatment.

These aspects of the action of picric acid were then tested in connection with activating agents other than heat. The agents used were ultra-violet irradiation (Heilbrunn and Wilbur, 1937), mixtures of sea water and isotonic (0.53 M) KCl (Wilbur, 1939), and mixtures of sea water and isotonic (0.35 M) sodium citrate

(Wilbur, 1941). Mixtures of KCl or citrate with sea water are denoted after the terminology of Wilbur (1941); thus a mixture of one volume of isotonic citrate and four volumes of sea water is called a "20 per cent sodium citrate mixture."

*Experiments showing inhibition by picric acid of various types of activation*

**Heat**—Of 15 experiments on the effect of picric acid on the sensitivity of eggs to heat, only one was inconsistent with the thesis that the acid inhibits the heat-activation. In these experiments, M/1000 picric acid was used, made up in sea water. Eight experiments proved useless, as the control percentages were too low to test any possible inhibition by the acid; the heat-sensitivity of these eggs is notoriously very variable between batches from different animals. The average of the seven experiments in which over 10 per cent of the control eggs responded is included in Table I, and shows a marked inhibition of the response by picric acid.

TABLE I

*Inhibition by picric acid of activation of Nereis eggs by various agents*

| Activating agent        | No. of expts. | Per cent activation<br>in absence of<br>picric acid | In picric acid, M/1000 |   |
|-------------------------|---------------|---|------------------------|---|
|                         |               |   | Per cent<br>activation | Per cent<br>with incipient<br>activation* |
| Heat                    | 7             | 57  | 4                      | 0   |
| KCl mixtures            | 18            | 96  | 4                      | 51  |
| Sodium citrate mixtures | 19            | 99  | 23                     | 28  |

\* As described on p. 147.

**KCl mixtures**—In fourteen experiments in which eggs were left indefinitely in a 25 per cent KCl mixture, and four similar experiments with a 50 per cent KCl mixture, almost always there was nearly 100 per cent activation in the absence of picric acid. When the acid was added to a concentration of M/1000, such activation occurred in only one instance; this case was distinctly unusual, as 74 per cent of the eggs were activated. Table I includes the averages for these experiments. However, in only 5 of the 18 tests was the breakdown of the germinal vesicle completely prevented. In the others, ordinary methods of observation (at 100 × magnification) did not reveal any certain change in appearance from the germinal vesicle stage, but a distinct nuclear outline could not be made out in many eggs. Compression of the eggs and higher magnification were necessary in counting these batches; the criterion employed was the visibility of a definite interface between the spherical nucleus and the cytoplasm. In the absence of this interface, the germinal vesicle was said to be broken down, even though no real alteration in the appearance of the egg was evident; the average percentage of the eggs so classified is presented in the last column of Table I. In these cells, the central nuclear region remained clear, the granular cortical opacity was retained, the oil droplets remained discrete and failed to migrate as in the activated eggs. None of the eggs of this type ever developed to a motile condition, or cleaved, or differentiated in any way. The appearance was as though nuclear breakdown had just barely begun when inhibition set in.

**Sodium citrate mixtures**—Complete or nearly complete inhibition of activation by picric acid was found in 12 of 19 experiments with citrate mixtures of 10–25 per cent. Of the other seven, two showed effective inhibition beyond the earliest stages of nuclear breakdown, as with the KCl mixtures (last column of Table I); one showed only moderate inhibition; only 4 of the 19 failed to show any significant inhibition. The averages are included in Table I. In these experiments, as in those with the KCl mixtures, the eggs were left in the activating agents indefinitely; counts were made with the eggs still in the various mixtures.

**Ultra-violet irradiation**—Only in relation to activation by ultra-violet rays did picric acid fail to exhibit an inhibitory effect. The presence of the acid (M/1000) in the sea water bathing the eggs did effectively prevent their activation by irradiation, but this action cannot be attributed to the effect of the acid on the eggs. Reduction of the depth of the egg-suspension to under 0.5 mm., so that the eggs are barely covered, permitted of ready activation by the rays, even in the presence of picric acid. The apparent inhibition in deeper samples is due to the absorption of the rays by the acid; the absorption spectrum of picric acid and picrates in salt solutions near neutrality (Eisenbrand and v. Halban, 1930; v. Halban and Litmanowitsch, 1941) is such that in any appreciable depth and concentration the supernatant fluid would prevent most of the active radiation from reaching the eggs, which always settle to the bottom of the dish. This interpretation is corroborated by the fact that a shield of picric acid in a quartz dish prevents any effect of ultra-violet rays on an underlying suspension of eggs in sea water.

**Fertilization by sperm**—Normal fertilization is completely inhibited in the solutions of acid used for the experiments above (in the range of M/1000). Addition of alkali to pH 8.0 did not affect this inhibition of fertilization. However, the removal of normally fertilized eggs to picric acid solutions within five minutes after fertilization (whether or not such solutions were alkalinized) did not appear to interfere with the normal development of the embryos; excellent survival and differentiation were obtained in the acid. Nevertheless, such embryos exhibited one outstanding anomaly: failure of the normal coalescence of the oil droplets. The oil in embryos growing in picric acid remained scattered as numerous discrete droplets; while under normal conditions these soon merge to form only a few, almost always four. The usual localization of the oil by migration (and segregation in cleavage) was not, however, altered in the course of development in picric acid solutions.

*Experiments showing synergism between various activators and the removal from picric acid to ordinary sea water*

**Heat**—Over 50 experiments tested the effect of baths in picric acid prior to exposure to heat in sea water. These showed a pronounced enhancement of the effects of the heat after the acid bath; not one showed a greater activation in the sample from sea water than in that from the acid. This relation between heat and removal from picric acid baths is shown in Figure 1. The synergistic action is evident only following the shorter baths, up to about 6 hours; since, after longer exposures to the acid, the mere removal to sea water was in itself sufficient to activate many eggs. The broken line curve in Figure 1 is made up from the combined data of all experiments involving removal of eggs from picric acid to sea water without further treatment. The other two curves on the same figure, however,

cover data from paired samples of eggs, and compare the effects of heat on eggs previously bathed in picric acid (in sea water) and on eggs from the same source not so treated.

The synergistic action was evident over a wide range of concentration of picric acid: from  $10^{-4}$  to just over  $10^{-3}$  M. The effects increased with increasing concentration, but above M/1000 the results became less reliable, so that M/1000 was used regularly, and is the only concentration for which data are reported. It is evident

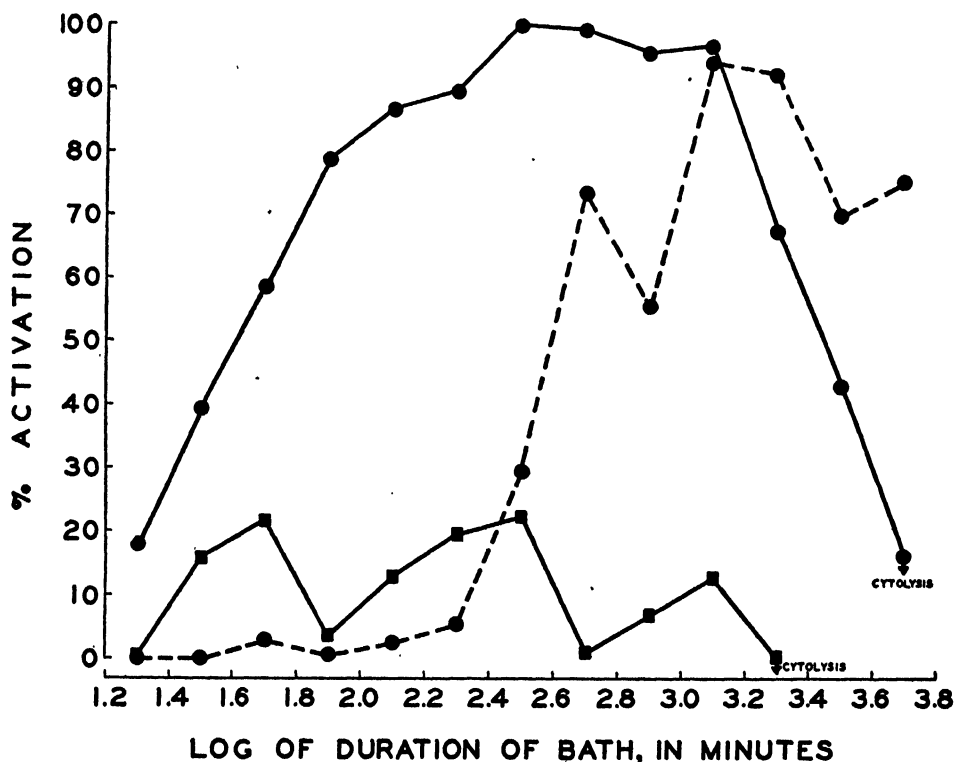


FIGURE 1. Relation of previous baths in picric acid to heat-activation of *Nereis* eggs.

Solid line connecting circular points—eggs heated after bath in M/1000 picric acid in sea water.

Solid line connecting square points—eggs heated after bath in sea water.

Broken line—eggs removed, unheated, from bath in M/1000 picric acid in sea water.

Each point is the average of all experiments performed in the logarithmic time interval denoted at the base-line. See text for further explanation.

from Figure 1 that the unfertilized eggs survived in the acid about twice as long as in sea water. Removal from sea water to the acid just prior to the expected onset of cytolysis (about 30 hours after removal from the animal) preserved the eggs as well as, but no better than, storage in the acid from the beginning.

**KCl mixtures**—The synergistic action of KCl mixtures and removal from picric acid to sea water was tested in 14 experiments, summarized in Figure 2(a). After 2–8 hours in the acid solutions, samples of eggs were removed to sea water and

to 5 per cent KCl mixtures; a control sample of the same batch of eggs kept in sea water was simultaneously exposed to the 5 per cent KCl mixture. This concentration of KCl is just below that necessary to induce regularly an appreciable percentage of response in ordinary eggs. Though the combined treatment was not in every case sufficient to activate the eggs, most experiments showed a pronounced synergism, and none showed a difference in the opposite direction. The response

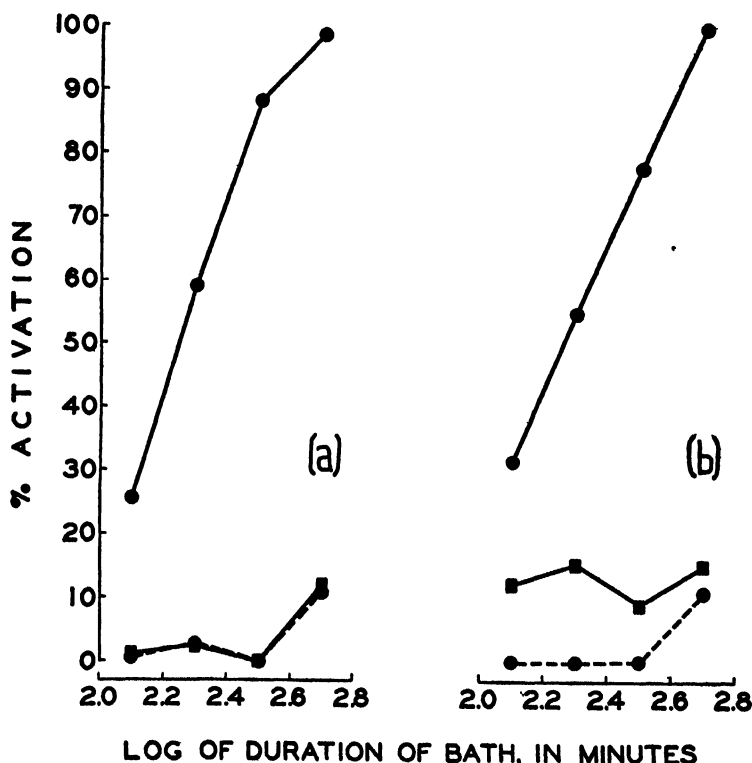


FIGURE 2. Relation of previous baths in picric acid to activation of *Nereis* eggs by (a) KCl mixtures, (b) sodium citrate mixtures.

Solid line connecting circular points—eggs treated after bath in M/1000 picric acid in sea water.

Solid line connecting square points—eggs treated after bath in sea water.

Broken line—eggs removed, untreated, from bath in M/1000 picric acid in sea water.

Each point is the average of all experiments performed in the logarithmic time interval denoted at the base-line. See text for further explanation.

of the eggs in these experiments, upon removal from picric acid baths to sea water, was somewhat less than average; thus the broken lines in the graphs in Figure 2 differ somewhat from the similar curve in Figure 1.

**Sodium citrate mixtures**—The same action was demonstrated with 10 per cent sodium citrate in place of the 5 per cent KCl mixture; 14 of 19 experiments showed a decided synergism. Five failed to show any significant difference between control and experimental. These failures were all among the shorter expo-

tures to the acid; the longer baths always resulted in increased sensitivity of the eggs to the citrate. This is illustrated clearly in Figure 2(b), which includes the data from all 19 experiments.

**Ultra-violet irradiation**—All attempts to show synergism between ultra-violet irradiation and removal from picric acid baths failed. A Uviarc mercury-vapor lamp, operating at 110 volts, 60 cycles, was used. The intensity of the radiation at the point at which the eggs were exposed was on the order of 6000 micro-watts per square centimeter.<sup>8</sup> Under such conditions, no significant differences could be found between the response to irradiation of eggs just removed from picric acid baths and those from sea water.

### DISCUSSION

The data illustrate three aspects of the action of picric acid in relation to activation of the eggs:

- (1) in the presence of certain concentrations of picric acid, heat-activation and chemical activation are prevented;
- (2) removal from the same concentrations of picric acid to sea water, after a short stay in the acid, acts synergistically with other activating agents in causing nuclear breakdown;
- (3) removal from the acid to sea water after longer stays in the acid leads to activation without assistance from other agents.

That fertilization and maturation of marine eggs is inhibited by acids is a common observation (Clowes and Greisheimer, 1920; Smith and Clowes, 1924; Tyler and Schultz, 1932; Tyler and Scheer, 1937); so that the inhibition by picric acid of artificial activation is not surprising. Similarly, the preservation of the unfertilized egg in picric acid against cytolysis and death is in accordance with many observations of this action of acids; some treatments were reported far more effective in this respect than picric acid appeared to be (Carter, 1931; Just, 1920; Smith and Clowes, 1924; Tyler and Horowitz, 1937a; Tyler and Dessel, 1939). The suggestion has even been made (Tyler, Ricci, and Horowitz, 1938) that the greater life-span of eggs in alcohol, dextrose, anoxic media, etc. (Gorham and Tower, 1902; Loeb, 1902; Loeb and Lewis, 1902; Lillie, 1931; Whitaker, 1937), can be explained in each case by the production of acids. The only odd aspect of the action of picric acid in this regard is that eggs stored in it for some time are subsequently over-sensitive to stimulators, and eventually are activated merely by removal to sea water. This was observed after a stay in the acid of as much as 70 hours. This is entirely dissimilar to the acid activation of starfish eggs, as investigated extensively by Lillie (1926, 1927, 1934, 1941). Lillie's exposures were of only a few minutes' duration, and the eggs were visibly altered while in the acid; a slightly prolonged exposure destroyed the eggs altogether. In picric acid, however, the eggs remain apparently unchanged for days, but immediately respond when removed to sea water.

This fact leads to the postulate that picric acid may react with, or in some way inactivate, an activating agent produced within the egg. Above a certain concentration, this agent would lead to activation of the egg; in still greater concentration, or under other conditions, to cytolysis. This agent is apparently being constantly

<sup>8</sup> Thanks are due to Dr. A. C. Giese for this measurement.

produced, and either diffuses from the egg, or is gradually destroyed as it is produced. But when picric acid is present within the egg, this agent is retained by the acid in an inactive form; when the egg is removed to sea water, the picric acid diffuses away, in turn releasing any acid bound with the activating agent. Thus the inhibition is removed, so that there is a sudden release of the accumulated activator within the egg, causing a response if the accumulation has been great enough.

Such a suggestion is in harmony with the synergism found between other activating agents and the removal from picric acid after exposures of lesser duration, and with the temporal pattern of the development of this synergism, as shown in Figures 1 and 2. The activating agents may be supposed to act by accelerating the production of the hypothetical activating substance; subliminal doses of these agents may then produce enough of the substance so that the added quantity released from the picric acid suffices to produce the response. That a still greater concentration may lead to cytolysis is indicated by the fact that less activation, with considerable cytolysis, is found when eggs are heated after a very prolonged exposure to picric acid, than when they are simply removed from the acid at the same time to sea water, without heating (Figure 1).

TABLE II

*Synergism between various activating agents in stimulation of Nereis eggs*

| Activating agents            |          |     | No. of expts. | Per cent activation |         |      |
|------------------------------|----------|-----|---------------|---------------------|---------|------|
| A                            | B        |     |               | A alone             | B alone | Both |
| Heat                         | Sodium   | 5%  | 9             | 24                  | 0       | 56   |
|                              | citrate  | 10% | 13            | 20                  | 4       | 83   |
| KCl 5%                       | Sodium   | 6%  | 1             | 0                   | 0       | 86   |
|                              | citrate  | 8%  | 1             | 0                   | 2       | 99   |
|                              |          | 10% | 1             | 0                   | 78      | 100  |
| Heat, without usual stirring | Stirring |     | 4             | 15                  | 0       | 39   |

The synergistic action indicates that at least to some extent activation is brought about through the same channels by all four agents: heat, KCl, sodium citrate, and removal from picric acid to ordinary sea water. Added evidence in this direction was obtained in experiments showing pronounced synergistic action between heat and citrate mixtures, and between KCl mixtures and citrate mixtures (Table II). As previously mentioned, stirring during exposure to heat had a pronounced enhancing action on the stimulatory effect of the heat, but stirring did not appear to act similarly in connection with the chemical activators. Mathews (1901) reported that Loeb and Fischer had been able to activate *Nereis* eggs by mechanical agitation alone, but all attempts in this direction failed.

Attempts to show synergism between ultra-violet irradiation and sodium citrate mixtures or removal from picric acid all failed; this is in keeping with the failure of picric acid to inhibit activation by ultra-violet rays. This may indicate that the radiation acts through a different mechanism than that involved in stimulation with the other agents. But under the conditions of the experiments the duration of

the exposures to ultra-violet was on the order of 30–60 seconds, much less than with the other types of activation; this difference in the rate of activation may be the entire explanation for the non-conformance of the experiments with this type of activation.

Heilbrunn (1925), Heilbrunn and Wilbur (1937), and Wilbur (1939, 1941) have presented several lines of evidence indicating that the breakdown of the germinal vesicle in the *Nereis* egg involves a reaction of calcium ions with the colloids of the protoplasm, and an associated set of changes in viscosity. Heilbrunn proposed that stimulating agents act by freeing calcium ions from combination (with lipoprotein) in the cell cortex, so that the calcium may react with the inner protoplasm; this interpretation of stimulation has been applied not only to the eggs of *Nereis*, but to cells in general. If such a mechanism is actually involved in the response of the *Nereis* egg, it might be expected that a penetrating acid would inhibit activation. The picric acid might acidify the protoplasm to the extent that the amphoteric protein molecules would become predominantly cations, with less Ca-binding capacity than previously. This interpretation would perhaps also explain the activation found upon removal of eggs from picric acid baths to sea water; the calcium freed from the cortex by the acid could react with the cell interior upon removal of the acid. Thus the same agent would act, in a sense, both as activator and as anesthetic. A serious difficulty with this explanation of the data lies in the fact that the eggs must be left in the acid for several hours, if they are to respond upon removal to sea water. This would require the assumption that the liberation by the acid of calcium ions from the cortex is a very slow process; or else that the acid continues to accumulate within the egg over a period of hours, quickly rising to the inhibitory concentration, but only after hours attaining the concentration active on the cortex. Neither of these assumptions is impossible, but both are rather involved.

If the action of picric acid were due to this proposed effect on the Ca-binding properties of proteins, other acids might be expected to act similarly. The action of other acids similar to picric, as regards pK and penetrating ability, has not yet been investigated; however, acetic, boric, and tannic acids have been used in experiments similar to those performed with picric acid. Acetic acid was used in concentrations from M/6000 to M/300; boric acid, from M/10<sup>6</sup> to M/5; and tannic acid, from M/10<sup>6</sup> to M/100; the upper limits of concentrations used were factors of the solubility and the effects of the acids on the eggs. Over M/1000, acetic acid often injured the eggs irreversibly, so that they were not fertilizable; this makes it difficult to evaluate cases of inhibition by acetic acid of activation, in the absence of tests for reversal of the effect. Ten to twenty experiments were performed with each acid in attempts to demonstrate synergism with heat, in the manner of picric acid; the duration of the baths ranged from 30 minutes to 24 hours. On a few occasions, acetic acid in concentrations around M/500 (concentrations not always innocuous) showed the synergistic action, but as often acted in the opposite manner (probably because of injury to the eggs). On one batch of eggs, M/10–M/20 boric acid also showed some synergistic action with heat, but this did not recur in similar experiments with other batches of eggs.

A further corroboration of the interpretation in terms of an activator-substance was sought in several attempts to accumulate the activator more rapidly by heating the eggs in picric acid, with subsequent release to sea water. In only 3 of 16 such

experiments was there markedly more activation in the eggs so treated than in those similarly exposed to the acid without application of the heat. However, none showed differences in the other direction; no data of any experiment thus far performed militates against the suggested scheme.

The completely reversible inhibitory action of picric acid is similar to the action of isotonic citrate in the experiments of Heilbrunn and Wilbur (1937) and Wilbur (1941). Since the citrate is presumed to act by removing calcium ions from solution by the formation of calcium citrate, there is a suggestion that perhaps calcium picrate is a similarly weakly dissociated salt. However, a few measurements of the electrical resistance of calcium picrate solutions showed that the equivalent conductance increased only slightly with dilution over the range  $n/100$ – $n/10,000$ . (The increase was in proportion to that found with  $\text{CaCl}_2$  in the same concentrations; the equivalent conductance of calcium citrate increased enormously with dilution over this range of concentration.) Thus the inhibitory action of picrate cannot be explained on the same basis as that applied to citrate inhibition.

In their extensive experiments on the peculiar action of many substituted phenols on the eggs of the sea-urchin, Clowes and Krah1 (1936), Krah1 and Clowes (1936, 1940), and Tyler and Horowitz (1937b, 1938) found picric acid one of only two or three inactive members of this group of compounds. Inhibition of cleavage was encountered only at concentrations around  $M/100$  or more, and the stimulation to respiration characteristic of this chemical group was lacking altogether. The calculations of Tyler and Horowitz showed that the concentration of dissociated picrate inside the cells was about  $100 \times$  that at which the related substances showed similar effectiveness. The same sort of relation was found for the other relatively inactive phenols. The latter should be tested in experiments similar to those with picric acid reported here. Such investigations might aid in deciding whether the action of picric acid is to be attributed to its acidity or to its particular molecular configuration.

Perhaps picric acid is unique in its combination of a low  $pK$  and a rapid rate of penetration into cells. The other phenols found to be exceptional (as regards inhibition of cleavage and stimulation to respiration) may share this combination of properties. Additional experimentation involving alteration of the picrate/picric acid ratio in the solution (through addition of  $\text{HCl}$  or  $\text{NaOH}$ ) is also suggested; such data might strongly indicate whether the acidity or the picrate itself is the active factor. On either basis, the present data clearly show that, while anesthetizing the eggs, this active factor constantly renders them increasingly sensitive to the removal of the anesthetization, and to subsequent stimuli. Proper interpretation of this fact might lead to a significant contribution to the understanding of the nature of stimulation.

#### SUMMARY

1. Germinal vesicle breakdown in *Nereis limbata* eggs, brought about by heat, or addition of  $\text{KCl}$  or sodium citrate to the sea water, was inhibited by the addition of picric acid at about  $M/1000$ .

2. After immersion for a few hours in  $M/1000$  picric acid in sea water, germinal vesicle breakdown occurred upon application of subliminal doses of heat,  $\text{KCl}$ , or sodium citrate.

3. After immersion for 6–70 hours, removal of the eggs from picric acid to ordinary sea water caused germinal vesicle breakdown.

4. Activation by ultra-violet irradiation did not conform in these relations to picric acid, under the conditions of the experiments.
5. These results are interpreted on the basis of a hypothetical activating substance produced within the egg, and inactivated or bound by picric acid.
6. The relation of picric acid to the calcium ion and the combination of calcium with protoplasmic proteins is considered, in an alternative explanation of the results.

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# A STUDY OF THE GOLGI APPARATUS IN CHICKEN GIZZARD EPITHELIUM BY MEANS OF THE QUARTZ MICROSCOPE

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The only fact about the Golgi apparatus that is universally accepted is that it is a cytoplasmic constituent of most cells which, after special fixation, blackens with silver nitrate or osmic acid. Controversies as to its structure, composition, function, and even its existence in the living cell, have been carried on continuously ever since Golgi originally described such a cellular constituent in 1898, totaling considerably over 2000 published papers. Mere descriptions of the blackened apparatus appear to be no longer fruitful. Furthermore, since cells must differ in order to carry on their specialized functions, warnings have been voiced against making hasty generalizations about all cells from studies on particular cells. The work reported in this paper pretends to nothing further than an analysis of certain features in one type of cell.

The Golgi apparatus in the lining of the chicken gizzard near its junction with the proventriculus is unusually spectacular and easily demonstrated. It can practically always be clearly shown after the usual osmic acid or silver nitrate techniques. Moreover, its size is enormous (Fig. 5). For these reasons chicken gizzard material is excellent for the study of the nature of the peculiar region of the cytoplasm where this network appears.

Previous studies have followed its changes in form during embryonic development of the gizzard (Hibbard, 1942). It can be demonstrated only in fixed material after appropriate impregnations. It can never be seen in living cells or in cells otherwise well fixed but not fixed by the usual methods for the Golgi apparatus. The only cytoplasmic inclusions which are rendered visible by methods other than silver and osmic impregnations in the general zone occupied by the Golgi apparatus, are vacuoles which may be stained vitally or postvitaly with neutral red, and occasional filamentous mitochondria. Hibbard (1942) has suggested that these vacuoles might be the antecedents of the typical Golgi network.

In an interesting series of papers, Worley (1943, 1944) has pointed out the high susceptibility of cytoplasmic inclusions, in many types of cells, to displacement or to changes in form and appearance with very slight changes in salt concentration in the surrounding fluid. Within ten seconds such distortions may take place so that quite different bodies from the original ones may be formed. Worley suggests, as Parat did nearly twenty years ago, that the fixed picture as it appears in sections may not at all resemble the living conditions.

Analysis of the Golgi apparatus by special types of illumination is not new. Monné in 1939 published two papers dealing with the appearance of the Golgi apparatus in *Helix* spermatocytes, using polarized light in one case and dark field illumination in the other, in order to demonstrate physical characteristics of difference

between the Golgi bodies and other cytoplasmic constituents. It must be remembered that his results apply to spermatocytes only and great care should be taken not to generalize them to apply to all Golgi bodies unless similar observations are made on other types of cells. There is a great difference between the spermatocyte Golgi apparatus and that in the glandular cells described in this paper, in staining reactions, susceptibility to deformation and the variety of methods by which it may be seen at all (Hibbard, 1945).

The present study was undertaken to determine, by means of ultraviolet microphotography, something about the nature of the cytoplasmic zone which becomes a complex network of blackened material after impregnation. It is not a study of the living cell, and may therefore be the analysis of an artifact. But if so it is one of extremely uniform occurrence in the chicken gizzard and one which undoubtedly has some precursor substance or some physical state of the material in the living cell which produces the localized black network in the fixed sections in perfectly regular fashion.

The technique employed was to make  $5\mu$  sections, either in paraffin or frozen, of material fixed in 7 per cent neutral formalin or in Da Fano, one of the fixatives nearly always successful in demonstrating the Golgi apparatus in gizzard epithelium. These sections were mounted on quartz slides under quartz coverslips and photographed with the quartz microscope using the  $2537\text{ \AA}$  line of mercury as the light source. The sections were unstained. Lavin (1943) has described the technique of ultraviolet microphotography.

Examination of the microphotographs shows that most cells have no peculiar tone variation in the zone in question (Fig. 3). However, in those cells with any apparent difference in the Golgi zone as compared with the rest of the cytoplasm, the Golgi zone appears somewhat paler (Figs. 1, 2, and 4).

It is known that nucleic acid has an absorption maximum of  $2600\text{ \AA}$  and proteins which contain tyrosine and tryptophane have an absorption maximum at  $2800\text{ \AA}$ . Nucleoproteins will have a maximum at some point intermediate. In order to show that these materials absorb in the ultraviolet region of the spectrum while in the solid state, microphotographs of globulin and of nucleic acid pellets were taken with the quartz microscope. They were cut and treated as if they were blocks of tissue. These photographs are reproduced in Figure 7 and Figure 8. It will be noted that while the nucleic acid is dark, the globulin remains pale. In a similar way microphotographs taken in the ultraviolet should demonstrate in tissues, with-

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FIGURE 1. Microphotograph, ultraviolet illumination;  $5\mu$  paraffin section after Da Fano fixation; no stain.

FIGURE 2. Microphotograph, ultraviolet illumination;  $5\mu$  paraffin section after 7 per cent formalin fixation; no stain.

FIGURE 3. Microphotograph, ultraviolet illumination;  $5\mu$  frozen section after 7 per cent formalin fixation; no stain.

FIGURE 4. Microphotograph, ultraviolet illumination;  $5\mu$  paraffin section after 7 per cent formalin fixation; no stain.

FIGURE 5. Microphotograph, visible light with green filter;  $5\mu$  paraffin section after Da Fano fixation followed by silver nitrate impregnation and reduction; no further stain.

FIGURE 6. Same as Figure 5.

FIGURE 7. Microphotograph, ultraviolet illumination; section of a pellet of globulin.

FIGURE 8. Microphotograph, ultraviolet illumination; section of a pellet of nucleic acid,

(In all figures, G—Golgi zone and N—nuclear region.)

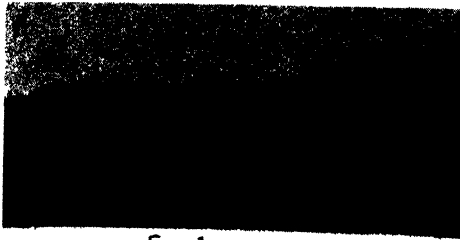


Fig. 1



Fig. 2



Fig. 3



Fig. 4



Fig. 5



Fig. 6



Fig. 7

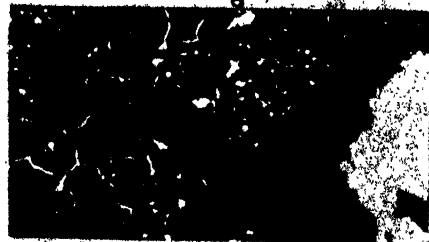


Fig. 8

out the necessity of staining, the presence of any substance of appropriate absorption maximum. Since the absorptive capacities of many organic tissue components are known and thus this method of demonstrating cell inclusions is a reflection of their chemical composition, we may say that the photographs present evidence that the Golgi zone does not contain appreciable amounts of nucleoproteins, nucleic acids or proteins containing tryptophane and tyrosine. The position of the Golgi apparatus in most cells is characteristically in close proximity to the nuclear surface, a position of possible physiological importance. This evidence that the region does not contain nucleoproteins or nucleic acid in large amounts if at all, is of some importance, particularly in view of the fact that most histological stains are in no sense chemical tests.

Our results also shed some light on the further question, which is of some interest: why does special fixation have to be practised before the subsequent impregnation will "take?" Examination of Figures 1, 2, and 4 will show that the Golgi zone is paler than the rest of the cytoplasm in many of the cells. In all probability this clear zone corresponds to the area blackened by silver nitrate. The curious fact is that this paler area may appear after formalin fixation whether the sections are imbedded in paraffin or frozen (Figures 2, 3, and 4), and also after Da Fano fixation without subsequent silver impregnation (Fig. 1). Silver impregnation applied after Da Fano fixation will produce a spectacular type of Golgi apparatus as shown in Figures 5 and 6, while exactly similar impregnation after neutral formalin as the fixative will produce only miscellaneous black granules throughout the cell with no greater blackening of the Golgi zone than of other regions. This seems to indicate that the Da Fano fixative either preserves some constituent lost in the formalin, or else adds itself to material already there, to make it reduce the silver in the conspicuous network. The identical absorption capacities of the region to ultraviolet light, whether the fixative be formalin or Da Fano, suggests a similarity in the quality of the fixed protoplasm. Why the Da Fano fixative should create a focal point exactly in the Golgi zone for the subsequent reduction of the silver, is not so clear. It may be that the Golgi zone is the site of aqueous vacuoles in the living cell, possibly containing highly dispersed materials such as proteins and lipoids, as found by Simpson (1941) after the freezing-drying technique. Both the work of Simpson and our own would indicate far less concentration of proteins in the Golgi zone than in the surrounding cytoplasm. During the process of fixation there may be a distortion of the zone as Parat thought, and more recently, Worley; and the distorted "apparatus" may be fixed by any fixative that coagulates the cytoplasm around it. The similar appearance of the apparatus as shown by ultraviolet photography, whether the fixative be formalin or Da Fano, shows that the reduction of the silver on or in the apparatus subsequent to fixation depends, in all probability, not on the fidelity of the whole cell's fixation but on the character of the fixative used.

In conclusion, these studies of cells by means of ultraviolet photography give certain negative information as to the material in the Golgi zone: it does not appear that it is nucleoprotein or nucleic acid, except possibly in greater dilution than in the rest of the cytoplasm. They also suggest that successful silver impregnation after one fixative and not after another may be due, not to less faithful fixation of the cell, but to a more direct relation between the fixative and the silver. Finally it

must be remembered that these studies were made exclusively on fixed material and there is much evidence that the morphology of the cellular constituents in such material does not coincide with that in living cells.

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# DILUTION MEDIUM AND SURVIVAL OF THE SPERMATOZOA OF ARBACIA PUNCTULATA.\* I. EFFECT OF THE MEDIUM ON FERTILIZING POWER

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## INTRODUCTION

The investigations in sperm physiology may be roughly divided into two principal aspects. First, there is the problem of the role of the sperm cell in fertilization. Second, there is the problem of the survival of spermatozoa as a fundamental condition for the survival of the species. These two aspects each have their own long lists of investigations.

The study of the sperm cell in fertilization has produced one outstanding theory. This is the Fertilizin Theory of Lillie (1914). The course of investigations, past and recent, shows that this theory, although not completely confirmed and probably in need of modification, has been found useful by many workers in the field. According to the theory, the male germ cell is the carrier of a substance, the "sperm receptor," which is functional in the fertilization process. This substance is thought to combine with "fertilizin," an egg secretion. The complex of sperm-receptor-fertilizin then reacts with an "egg receptor" to form a three-way complex in the egg. The formation of this ternary complex initiates the fertilization reactions of the egg.

The study of sperm senescence, in contrast to the above, has yielded results which are, at best, unsatisfactory. Gray (1928a and b), who investigated the changes in metabolism of sperm under various conditions, reported that sperm in highly concentrated condition have a very low rate of respiration. If diluted, the sperm show a burst of metabolic activity. The greater the dilution, the more intense is this burst of action, although of shorter duration. Gray advanced the hypothesis that a large part of the sperm cell's internal supply of fuel was used up in the first burst of energy, so that the greater its intensity, proportionately shorter became the life of the spermatozoön. The initial burst of activity was in turn determined by the available "free space" in which the sperm cell could move, that is, by the dilution. In the limited space available to each cell in the concentrated suspensions, the sperm cell was only incompletely activated, and, hence, its life was prolonged.

This explanation cannot be applied without certain limitations. If it were, a single spermatozoön placed in an infinitely large volume of diluent would end its metabolism instantly. Further, "mechanical crowding" as an explanation is applicable only to the translatory or vibratory activity of the sperm and not to the respiratory activity. With all the known variables, such as oxygen and carbon dioxide tensions, rigidly controlled, Gray's evidence shows that when "free space" is available the rate of sperm respiration increases. "Mechanical crowding" is thus

\* Work done as part of the requirement for the degree of Doctor of Philosophy.

not an explanation for the changes in respiratory rate but a description of the conditions under which the respiratory rate is low. That is, it is logical to state that sperm are quiescent because they are forced to be immobile, but it is not logical to state that sperm respire at a high rate because they are no longer forced to be immobile. Such a statement has implications of teleology. There must exist an unknown factor which, under conditions of dilution, brings about the increased respiration of sperm. Undiluted sperm, therefore, must be a system composed of the cells plus the unknown factor. Dilution of the system, not the dilution of cells alone, brings about the respiratory activity of the spermatozoa.

The foregoing review shows that Lillie's fertilization studies have indicated the existence of a substance that determines the fertilizing power of the sperm cell. The review shows, too, that Gray's work has neglected one variable, the sperm cell medium, or a factor in that medium which affects the duration of metabolic activity of the sperm cell. It is the purpose of this research to present evidence for the existence of a single factor that influences the conservation of fertilizing power by sperm and the respiratory activity of sperm. The work is presented in two sections, the first section dealing with the fertilizing capacity of sperm, and the second with the respiratory activity of sperm.

The author is greatly indebted to Dr. Daniel Mazia for his guidance and helpful suggestions.

#### MATERIALS AND METHODS

The materials used in the series of experiments to be described were the germ cells of the Atlantic sea-urchin, *Arbacia punctulata*. The general methods and precautions outlined by Just (1939) were followed carefully. To obtain the germ cells, the urchins were thoroughly washed in running sea water and running tap-water, after which they were dried carefully with clean cheese-cloth. A cut around the oral region disclosed the sex of the animal. If male, the sperm exuding from the genital pores were received in a dry stender dish; if female, the animal was allowed to shed the eggs into a stender dish filled with sea water.

The sperm suspensions for the earlier experiments were made according to the "drop" method of Lillie (1913). For greater precision in later experiments, sperm were "packed" by centrifugation at 3500 r.p.m. for 30 minutes. These packed sperm cells were drawn into a calibrated capillary tube. The tip of the capillary was wiped clean, and the contents were used to make the sperm suspension. The capillary was calibrated by taking up the same volume of re-distilled mercury and weighing the mercury accurately.

As a check on the constancy of this method, sperm counts were made. A unit quantity of packed sperm was suspended in one cc. of sea water, shaken thoroughly, and 0.01 cc. of Bouin's fixative added. This suspension was diluted one hundred times, and the number of sperm present counted in a haemocytometer chamber. The results are given in Table I, and it was found that the greatest deviation from the average was in the order of 6 per cent, a constancy not attainable by the "drop" method.

The seminal fluid used in the experiments was collected simply by drawing off the supernatant fluid from the packed "dry" sperm after centrifugation.

The egg suspensions were made by washing the eggs several times in sea water and allowing them to settle in the dish by force of gravity. Equal samples of the

TABLE I

*Sperm count, using 0.00155 cc. packed sperm per cc. of sea water, diluted 100 times*

| Suspension | Number squares counted | Total counted | Average number per sq. | Conc. of packed sperm per cc. |
|------------|------------------------|---------------|------------------------|-------------------------------|
| No. 1      | 32                     | 386           | 12.0                   | $3.06 \times 10^{13}$         |
| No. 2      | 32                     | 346           | 10.8                   | $2.76 \times 10^{13}$         |
| No. 3      | 32                     | 379           | 11.8                   | $3.02 \times 10^{13}$         |
| No. 4      | 32                     | 360           | 11.2                   | $2.86 \times 10^{13}$         |
| No. 5      | 32                     | 390           | 12.1                   | $3.09 \times 10^{13}$         |
| No. 6      | 32                     | 359           | 11.2                   | $2.86 \times 10^{13}$         |
| Average    |                        |               |                        | $2.94 \times 10^{13}$         |

settled eggs were diluted in varying amounts of sea water, mixed to give homogeneity, and aliquots were removed with a calibrated pipette. From the number of eggs present per unit length, the total number of eggs could be calculated. One drop of a suspension of suitable egg-concentration was placed in 5 cc. of sea water. Generally, the number of eggs in one insemination test was 750–1000. As Lillie (1915a) had shown, variations of this order in the total number of eggs used in the inseminations do not affect the final results appreciably. Since a fresh egg suspension was used for the insemination tests at any one time, the tests at two different times used different suspensions whose concentrations varied somewhat, so that the results were possibly not comparable. Those tests run at any one time used the same egg suspension, and, therefore, the results were comparable to each other.

For the insemination, a unit quantity of the sperm suspension in a pipette was carefully squeezed out over the eggs, and the whole dish gently and uniformly stirred. For determination of fertilizing power, the percentage of eggs activated was calculated by counting a minimum of 200 eggs.

Widely diverging types of experiments were made in the course of this investigation, each type entailing its own methods and techniques. Because of this, other methods and techniques will be described in connection with particular experiments. Each typical experiment to be presented in the following section was one of a minimum of five experiments all giving similar results.

## EXPERIMENTS AND RESULTS

### *The seminal fluid factor and the survival of sperm*

Past researches have shown that sperm in the undiluted condition freshly-exuded from the testes are immobile, and that the sperm manifest intense activity upon dilution with sea water. Subsequently, the fertilizing power of the sperm cells declines sharply and within a relatively short time. Workers in the past had diluted sperm fresh from the testes with sea water. Since the medium seemed to be a variable in this type of dilution, and since sperm cells in the testes were suspended in a liquid medium, a factor influencing the fertilizing capacity of sperm cells was sought in the seminal fluid.

To examine the effect of the seminal fluid on the fertilizing power of sperm, a series of experiments was done using sperm suspensions of the same concentration in seminal fluid and in sea water. These suspensions were then tested at different time intervals for their fertilizing power. In the experiment shown in Table II, a 0.4 per cent sperm suspension (according to the terminology of Lillie) was used. One drop of the suspension was used to inseminate 750–1000 eggs. The formation of the fertilization membrane was used as the index of activation of the egg.

The effect of the seminal fluid in promoting the survival of the sperm was apparent even after five hours, and after 12 hours, when the sperm in sea water were completely non-functional, a large number of those in the seminal fluid were still capable of bringing about activation. At each test, microscopic observation revealed that the per cent activation of eggs was approximately directly proportional to the number of motile sperm.

TABLE II

*Activation of eggs by sperm suspensions of 0.4 per cent concentration in seminal fluid and sea water*

| Medium     | Per cent activation |         |        |        |        |         |
|------------|---------------------|---------|--------|--------|--------|---------|
|            | 10 a.m.             | 11 a.m. | 3 p.m. | 5 p.m. | 8 p.m. | 10 p.m. |
| Sea water  | 100                 | 100     | 46     | 22     | 0–2    | 0       |
| Sem. fluid | 100                 | 100     | 99     | 100    | 99     | 95      |

The maintenance of fertilizing power of the sperm cells was a function specific for the seminal fluid. Experiments were made using the perivisceral fluid as the suspension medium. The perivisceral fluid was found to have a toxic effect on the retention of fertilizing power by sperm.

It seemed clear that in the seminal fluid an unknown factor was enabling the sperm to retain their fertilizing power for a long period of time. In view of the work of Cohn (1918), a check of the effect of pH became necessary. The pH of the seminal fluid was measured electrometrically with McGinnis' electrode. A number of such measurements showed the pH of seminal fluid to vary between 7.6 and 7.9.<sup>1</sup> Experiments were done comparing the survival of sperm in seminal fluid and sea water acidified to the same pH as the seminal fluid sample.

In the same experiments, another chemical property of the seminal fluid was investigated, namely, the heat-sensitivity. A sample of the seminal fluid in a test tube was heated at 100° C. for ten minutes, the seminal fluid allowed to cool to room temperature, and this heated seminal fluid was tested for its effect on the survival of sperm.

The results of experiments are summarized in Table III. The dilution used was one drop of centrifuged sperm to 5 cc. of medium. The pH of this seminal fluid sample was 7.72; the sea water (pH 8.0) was acidified to 7.7 by the addition of 11 drops of 0.1 N HCl to 100 cc. of sea water. All the suspensions were made at 5 p.m.

The results showed that acid sea water maintained the fertilizing power of the sperm only slightly longer than normal sea water and not nearly so long as the

<sup>1</sup> Done by Mr. M. E. Smith, of the MBL staff.

TABLE III

*The effects of pH, heated seminal fluid on the survival of sperm,  
as shown by time measurements of the fertilizing power*

| Medium       | Per cent activation |        |            |        |         |
|--------------|---------------------|--------|------------|--------|---------|
|              | 5 p.m.              | 9 p.m. | 10:30 p.m. | 4 p.m. | 10 p.m. |
| Sea water    | 100                 | 100    | 14         | 0      | 0       |
| Sem. fluid   | 100                 | 100    | 100        | 97     | 73      |
| Heated fluid | 100                 | 0      | 0          | 0      | 0       |
| Acid s.w.    | 100                 | 100    | 40         | 2      | 0       |

seminal fluid. The heated seminal fluid, on the other hand, had clearly lost the function of promoting the survival of the sperm cells. It was evident that pH was not the effective factor in the seminal fluid and that the effective factor was heat-sensitive.

This heat sensitivity led to the suspicion that the unknown factor was protein. To test this hypothesis, the seminal fluid was saturated with ammonium sulfate. A faintly rose-colored precipitate resulted from this treatment. This precipitate was filtered off, and the residue on the filter paper dissolved in a volume of sea water equal to the original volume of seminal fluid. The sea water containing the residue was then dialyzed against fresh changes of sea water in the refrigerator for 30 hours. The dialyzing membrane was commercial sausage skin (Cenco). This treatment removed the ammonium sulfate. The liquid inside the dialysis bag, essentially an artificial seminal fluid, was then used as the suspending medium for the sperm.

As controls for this experiment, various other media were used to suspend equal concentrations of the same sperm sample. For the first of these, the filtrate of the seminal fluid (seminal fluid minus the precipitated material) was also dialyzed against sea water for the same length of time as the residue solution, and this "dialyzed filtrate" was used as a suspending medium for the sperm. Normal sea water, acid sea water, and natural seminal fluid were also run as controls. The dilution used was one drop of centrifuged sperm to 10 cc. of medium, and the pH was carefully checked in each case.

The results (Table IV) showed that spermatozoa in the "artificial seminal fluid" retained their fertilizing power nine hours longer than did the sperm in sea water. From the data, it was concluded that the seminal fluid factor was precipitable with ammonium sulfate and non-dialyzable. The earlier conclusion as to the negligible effect of pH was confirmed in this experiment.

The idea of the seminal fluid factor's being protein seemed to be borne out and warranted an analysis of the seminal fluid for its protein content, along with determinations of other physical and chemical properties. For determination of protein, Folin's micro-Kjeldahl with direct Nesslerization was used, the solutions being compared in a photoelectric colorimeter. The results showed 2.5 mg. protein per cc. of 100 per cent seminal fluid. The pH of the seminal fluid was found to vary between 7.6 and 7.9 as compared to the pH of sea water, which varied from 7.9 to 8.1.<sup>2</sup> The freezing point of seminal fluid was  $-1.715^{\circ}$  C. as compared to that of

<sup>2</sup> Done by Mr. M. E. Smith, of the MBL staff.

TABLE IV

*The effects of various media on the survival of sperm, as shown by insemination tests*

| Medium         | pH  | Per cent activation |          |           |           |           |           |
|----------------|-----|---------------------|----------|-----------|-----------|-----------|-----------|
|                |     | 0.5 hrs.            | 5.5 hrs. | 10.0 hrs. | 15.5 hrs. | 24.0 hrs. | 26.0 hrs. |
| Sea water      | 8.0 | 98                  | 92       | 49        | 13        | 0         | 0         |
| Acid sea water | 7.7 | 98                  | 87       | 72        | 68        | 0         | 0         |
| Sem. fluid     | 7.6 | 100                 | 97       | 100       | 100       | 98        | 35        |
| Dial. residue  | 7.8 | 99                  | 96       | 98        | 85        | 19        | 4         |
| Dial. filtrate | 7.8 | 93                  | 80       | 20        | 5         | 0         | 0         |
| Eggs tested    |     | 98                  | 98       | 100       | 100       | 98        | 100       |

sea water, which was  $-1.892^{\circ}\text{C}.$ <sup>3</sup> Chloride analysis showed the sea water to contain 0.508 moles per liter, while the seminal fluid contained 0.590 moles per liter.<sup>4</sup> Analysis for glucose (reducing sugar) showed the seminal fluid to contain less than 10 gamma in 5 cc.

These results suggested as one possibility that the action of seminal fluid on the sperm could be attributed to the osmotic pressure difference between the seminal fluid and the sea water. The demonstrated heat-sensitivity of the seminal fluid factor, however, ruled this possibility as unlikely, as did the prolonged dialysis of the last experiment given, for such treatment would equalize the osmotic pressure of the seminal fluid with that of the sea water.

The difference in chloride content between the seminal fluid and sea water was not considered as a factor in prolonging the fertilizing power of the sperm cells. The prolonged dialysis described earlier would have equalized the chloride concentration of the sea water and the "artificial seminal fluid" of Table IV, yet these two media had markedly different effects upon the sperm cells. Also, the demonstrated heat sensitivity of the seminal fluid factor indicated that it was not chloride.

The effective seminal fluid factor therefore seemed to be protein, but protein, by its presence, would establish a colloidal osmotic pressure which might be the agency acting on the sperm.

In Table IV, it may be noted that the "dialyzed residue" was not as effective as the natural seminal fluid. There are several possible explanations. First, during the prolonged dialysis, some of the protein may have been denatured, a point to be checked in future investigations. Second, the concentration of the factor in the "artificial seminal fluid" was probably not equal to that in the natural medium, due to some loss of protein in handling, and difficulties in volume control in dialysis.

At this point, attention should be called to the fact that still another possibility existed as to the manner in which the seminal fluid functions. This was the question of nutrition of the sperm by the seminal fluid. This question will, however, be taken up in the discussion.

There remained one mode of action of the seminal fluid factor hitherto uninvestigated. The results of the experiments already described validated the as-

<sup>3</sup> Done by Dr. Jay A. Smith, of the MBL staff.

<sup>4</sup> Done by Mr. J. Weissiger, of the MBL staff.

sumption that the seminal fluid factor acted in some manner upon the surface of the sperm cells.

Observations made during attempts to measure sperm activity in a capillary tube showed spermatozoa to be positively thigmotropic to glass surfaces. At the instant of contact, the spermatozoön lost a large part of its activity and rotated slowly about its point of contact. The observation seemed to show the presence of a surface active substance on the head of the spermatozoön. This fact, previously observed by Buller (1902), led to the following experiment.

Three suspensions of sperm of equal concentration were made in sea water. Suspension No. 1 was left untreated. Glass powder was added to suspensions No. 2 and No. 3. All three suspensions were shaken simultaneously and placed in the refrigerator, where the powdered glass was allowed to settle for three hours. Insemination tests were run to determine the relative sperm populations in these three suspensions. Qualitative microscopic observations on sperm population were also made at each dilution of the original suspensions as a check.

As shown in Table V, the results indicated that the sperm population in the second and third suspensions was greatly reduced, a result confirmed by microscopic observation. It was possible that the glass powder injured a large part of the total sperm population, but the absence of significant numbers of injured sperm seemed to indicate that the glass powder removed the missing sperm by adhesion.

TABLE V

*Activation of eggs by progressive dilutions of sperm suspensions treated with glass powder as compared to untreated sperm suspension*

| Suspension | Undiluted | 1:1 Dilution | 3:1 Dilution |
|------------|-----------|--------------|--------------|
| No. 1      | 100       | 100          | 100          |
| No. 2      | 96        | 61           | 27           |
| No. 3      | 100       | 75           | 35           |

A similar experiment was made to test the surface activity of seminal fluid protein, since the proposed surface-action implied the identity of sperm-surface-substance and seminal fluid protein. A sample of seminal fluid was divided into three portions. Portion No. 1 was left as the untreated control. Glass powder was added to portion No. 2, the portion shaken thoroughly, and the glass powder filtered off with Whatman No. 5 filter paper. Portion No. 3 was shaken three times, each time with fresh glass powder and filtered free of glass each time. These seminal fluid portions were then used to make sperm suspensions of equal concentration and tested for the maintenance of the fertilizing power. The results are given in Table VI.

Clearly, the glass powder removed the sperm-longevity factor from the seminal fluid, so that seminal fluid protein, too, seemed to be surface-active on glass. Although the experiments of Tables V and VI did not completely establish the identity of the seminal fluid factor and the substance on the surface of the sperm, they did show that both substances were apparently surface-active.

In furtherance of this line of thought, experiments were made to learn whether sperm in sea water gave off their surface substance into the surrounding medium.

TABLE VI

*Removal of the factor from seminal fluid with glass powder*

| Medium        | Per cent activation |          |          |           |           |           |           |
|---------------|---------------------|----------|----------|-----------|-----------|-----------|-----------|
|               | 0.0 hrs.            | 4.0 hrs. | 9.5 hrs. | 12.0 hrs. | 14.5 hrs. | 24.0 hrs. | 28.0 hrs. |
| Sea water     | 100                 | 100      | 78       | 65        | 40        | 0         | 0         |
| Portion No. 1 | 100                 | 100      | 100      | 100       | 100       | 100       | 95        |
| Portion No. 2 | 100                 | 100      | 100      | 80        | 54        | 5         | 0         |
| Portion No. 3 | 100                 | 99       | 1        | 0         | 0         | 0         | 0         |
| Eggs tested   | 100                 | 100      | 100      | 100       | 100       | 100       | 100       |

In one type of experiment, a heavy suspension of sperm in sea water was allowed to stand for several hours. The sperm were then removed by centrifugation and the supernatant fluid tested as a sperm medium. In another type of experiment, the above procedure was repeated several times, the supernatant fluid used to support a fresh sample of sperm after each centrifugation. After the final centrifugation, the supernatant fluid was tested for its effect on fresh sperm. In all cases, the results were negative. Such "sperm washings" had neither a detrimental nor favorable effect on the maintenance of the fertilizing power of the sperm.

There remained one other point of investigation in the survival time of spermatozoa. Observations had shown that seminal fluid protein, even in low concentration, was effective in maintaining spermatozoa. Gray (1928a) had postulated a "mechanical crowding" effect as the primary factor in the survival of sperm. Since he used "dry" sperm, which was composed of about 60 per cent seminal fluid, there arose the possibility that the longer survival of the more concentrated sperm had as its cause, not "mechanical crowding," but the larger amounts of seminal fluid protein carried over in the "dry" sperm. A test of this possibility followed.

A sperm suspension in seminal fluid was made by suspending 0.025 cc. of packed sperm in one cc. of seminal fluid. A second suspension was made by taking 0.2 cc. of the first suspension and adding it to another one cc. sample of seminal fluid. This serial dilution was repeated twice more, to make four sperm suspensions, all in seminal fluid. The operation was carried out quickly, the last suspension made within a minute of the first. The final concentrations of the four suspensions were, in Lillie's terminology, approximately 5 per cent, 1 per cent, 0.2 per cent, and 0.04 per cent, since packed sperm contained approximately twice the amount of sperm per unit volume as did the "dry" sperm used by Lillie. The insemination tests were made at the same dilution, each of the more concentrated suspensions being diluted to the lowest concentration of 0.04 per cent. One drop of this final suspension was used to inseminate the eggs. The results are given in Table VII.

A study of these results as compared to those of Gray showed that, even though Gray's results might be partly explained as the action of seminal fluid protein, "mechanical crowding" did seem to play a part in determining the life-span of the spermatozoa. However, it may be pointed out that this "crowding effect" seems to be non-linear in relation to the concentration, and is most apparent at extreme dilutions.

TABLE VII

*The effect of concentration on the survival of sperm in seminal fluid*

| Suspension concentration | Per cent activation |          |          |           |           |           |
|--------------------------|---------------------|----------|----------|-----------|-----------|-----------|
|                          | 0.5 hrs.            | 4.0 hrs. | 7.5 hrs. | 18.0 hrs. | 23.0 hrs. | 30.0 hrs. |
| 5 per cent               | 88                  | 90       | 78       | 77        | 85        | 91        |
| 1 per cent               | 81                  | 85       | 83       | 79        | 83        | 72        |
| 0.2 per cent             | 90                  | 92       | 84       | 80        | 49        | 13        |
| 0.04 per cent            | 92                  | 95       | 86       | 45        | 10        | 0         |

*The seminal fluid factor and its role in fertilization*

In the course of the preceding experiments, sea water suspensions of sperm used to test the eggs showed a contrasting behavior as to fertilizing power. The individual spermatozoon in seminal fluid appeared to have a greater fertilizing power than the spermatozoon in sea water. An experiment was devised to investigate this more closely.

A volume of 0.025 cc. of packed sperm was suspended in one cc. of seminal fluid. Immediately after the suspension was made, one drop of the suspension was used to inseminate approximately 1000 eggs. Serial dilutions were made as for the previous experiment, but as each new suspension was made, one drop was used to inseminate approximately 1000 eggs. A sea water control was run, dilution and inseminations being made in the same way (Table VIII).

TABLE VIII

*A comparison of the fertilizing power of sperm in seminal fluid and sperm in sea water*

| Medium     | Per cent activation |              |              |              |
|------------|---------------------|--------------|--------------|--------------|
|            | 1st dilution        | 2nd dilution | 3rd dilution | 4th dilution |
| Sea water  | 100                 | 97           | 37           | 12           |
| Sem. fluid | 100                 | 99           | 100          | 81           |

The results proved that there was a strong difference in the fertilizing power of the sperm cells in seminal fluid as compared to those cells in sea water. This difference became even more pronounced when the original concentrated suspensions were allowed to stand for ten hours, as shown in Table IX. Only the most concentrated suspensions in the seminal fluid and the sea water were kept. The dilutions were made anew.

The interpretations of these results were rather complex and will be discussed in a later section.<sup>5</sup>

The apparent increased fertilizing power of the sperm in the seminal fluid indicated that seminal fluid factor might be directly concerned with the fertilization process. It was recalled that Lillie (1915) had given as one of the criteria for the "sperm receptor", the power to "bind" agglutinin from the egg. Lillie meant by this

<sup>5</sup> See page 175.

TABLE IX

*A comparison of the fertilizing power of sperm in seminal fluid and sea water after 10 hours*

| Medium     | Per cent activation |              |              |              |
|------------|---------------------|--------------|--------------|--------------|
|            | 1st dilution        | 2nd dilution | 3rd dilution | 4th dilution |
| Sea water  | 100                 | 86           | 7            | 0            |
| Sem. fluid | 100                 | 100          | 98           | 79           |

that if the agglutinin were treated with the "sperm receptor" solution (here the seminal fluid, presumably), the action of the agglutinin on the sperm would be greatly reduced. This experiment was done, with the expectation that, if the seminal fluid factor and the "sperm receptor" were one and the same, the agglutinating action of the egg secretion would be reduced.

A series of dry watch glasses was arranged. In the first, two drops of seminal fluid and two drops of egg-water were thoroughly mixed. Two drops of this mixture were then removed to the next watch glass and diluted with two drops of sea water. This treatment was repeated down the series. For the control, sea water was used instead of seminal fluid. For the test, a drop of a standard sperm suspension (0.00155 cc. packed sperm per cc. of sea water) was placed in the watch glass, out of contact with the mixture. The watch glass was then placed under the objective of the microscope, the two liquids (sperm suspension and sea water-seminal fluid mixture) shaken together, and the reaction of the sperm noted. In the following table, + indicates a positive agglutination, - a negative agglutination, and  $\pm$  uncertain. The number of + symbols indicates the intensity of the reaction.

TABLE X

*The agglutination reaction induced by dilutions of egg-water-seminal fluid mixtures, as compared to those induced by egg-water-sea water mixture of the same dilutions*

| Dilution | Sea water<br>egg-water | Sem. fl.<br>egg-water |
|----------|------------------------|-----------------------|
| 1        | +++                    | +++++                 |
| 1/2      | +++                    | +++++                 |
| 1/4      | +++                    | +++                   |
| 1/8      | ++                     | +++                   |
| 1/16     | +                      | ++                    |
| 1/32     | $\pm$                  | +                     |
| 1/64     | -                      | +                     |
| 1/128    | -                      | $\pm$                 |

Instead of having its action on the sperm reduced, the results revealed that egg-water treated with seminal fluid had, if anything, a more powerful agglutinating power than the sea water-treated egg-water. In any event, the agglutinating power was not reduced. The only conclusion possible from these results seemed to be that the seminal fluid factor was not the "sperm receptor" of Lillie.

However, the data given indicated that the sperm reaction in the seminal fluid-egg-water mixture was more intense than the corresponding reaction of sperm in the egg-water sea water mixture. This phenomenon was put to a quantitative test.

Standard suspensions of sperm were made in seminal fluid and in sea water. The concentration was 0.00155 cc. packed sperm per cc. of medium. These suspensions were allowed to stand at room temperature (25° C.). At intervals, a drop from either one or the other of the suspensions was placed on a watch glass, out of contact of a mixture of one drop of egg-water and two drops of sea water. The liquids were shaken together under the microscope, and the time of agglutination (from onset to reversal) was taken with a stop-watch. The results are summarized in Table XI.

TABLE XI

*The agglutination time of sperm suspended in seminal fluid as compared to that of sperm suspended in sea water*

| Time Tested<br>p.m. | Agglutination time in seconds |                        |
|---------------------|-------------------------------|------------------------|
|                     | Sperm in sea water            | Sperm in seminal fluid |
| 3:00                | 90                            | 120                    |
| 3:06                | 63                            | 69                     |
| 3:10                | 61                            | 115                    |
| 3:18                | 82                            | 98                     |
| 3:40                | 49                            | 111                    |
| 3:44                | 53                            | 95                     |
| 3:50                | 47                            | 91                     |
| 3:57                | 63                            | 90                     |
| 4:05                | 61                            | 97                     |
| 4:40                | 91                            | 86                     |
| 4:50                | 86                            | 75                     |
| 5:15                | 34                            | 120                    |
| 5:30                | 71                            | 115                    |
| 7:30                | 75                            | 76                     |

The data show that, on the average, the sperm in the seminal fluid remained agglutinated for a longer time than the sperm in sea water. Although the results showed wide variation, the contrast between the two sperm suspensions was quite striking. From the results, it seemed reasonable to conclude that seminal fluid had changed the sperm surface in such a way as to bring about a stronger reaction with agglutinin.

#### DISCUSSION

##### *The seminal fluid factor and sperm motility*

Gray (1928a) observed that the motility of the sperm of *Echinus miliaris* was in no way impaired when suspended in seminal fluid, and he stated conclusively that the seminal fluid possessed no chemical or physical properties inhibiting sperm motility. He prepared the seminal fluid, which he called "testicular plasma," by strong centrifugation of the "dry sperm," the same method employed in this investigation.

The experiments and observations of the present study confirm Gray. The earlier results of the work, given in a preliminary note (Hayashi, 1940), showed that the sperm of *Arbacia punctulata* were motile in seminal fluid, with an intensity of movement at least equal to that exhibited by sperm in sea water. Moreover, this motility persisted for a longer time in the former medium. That sperm are active in seminal fluid was confirmed by respiration studies (results to be given in a subsequent report), for it was found that the respiratory activity of sperm was maintained

at a higher level for a longer time in seminal fluid than in sea water. Therefore, it may be stated conclusively that sperm cells of *A. punctulata* and *E. miliaris* are fully active in seminal fluid.

The observations and conclusions of Southwick (1939a) were found to be in conflict with these results. This worker found that sperm of *Echinometra subangularis* were immobile when suspended in the seminal fluid of the same species. He concluded that there was present in the seminal fluid a substance which inhibited the activity of the sperm.

Hartman (1940) and Hartmann, Schartau, and Wallenfels (1940) confirmed Southwick on the presence of the inhibiting factor not only in the seminal fluid, but also in the sea water that had contained large numbers of spermatozoa. Their work, however, was done with the sperm of *Arbacia pustulosa*. In addition to confirming Southwick, Hartmann et al. stated that the function of the inhibiting factor was to neutralize echinochrome A, a sperm-activating substance from the egg.

For several reasons, the conclusions of these workers do not seem to be justified. In the first instance, Southwick's own observations reveal that freshly-exuded "dry sperm" possess an intense vibratory activity, an apparent contradiction to his own conclusion. This activity is lost after a few minutes. A number of investigators have published observations pertinent to these phenomena. Thus, Harvey (1930) showed that sperm of *Arbacia punctulata* in oxygen-free sea water were immobile; when oxygen was introduced the sperm regained their motility. Lillie (1913) demonstrated that sperm of *Nereis* and *Arbacia* lost their motility in the presence of carbon dioxide. Dungay (1913), using *Nereis* and *Arbacia*, Fuchs (1914) with *Ciona intestinalis*, and Cohn (1918) with *A. punctulata* proved that acid media had a deleterious effect on sperm. Finally, Carter (1931) working with *Echinus esculentus* and *Echinus miliaris*, and Taylor and O'Melveny (1941) with *Strongylocentrotus purpuratus* and *Lytechinus anamesus* obtained experimental proof that acid conditions lowered the respiratory activity of sperm.

In view of the results of these investigators, the brief activity of the sperm noted by Southwick seems to be attributable to the newly-made contact of the sperm with oxygen upon shedding. The subsequent inactivation of the sperm has its probable explanation in the acid conditions induced by the carbon dioxide production of the sperm.

Furthermore, the papers of Southwick and the Hartmann school yield no figures on the pH of the media used by these workers, nor do their texts give any evidence that this factor had been controlled. In addition, the conclusions of Hartmann et al. concerning the effect of echinochrome have not been confirmed by the experiments of Tyler (1939b) and Cornman (1940, 1941). The former worker found that neither echinochrome nor spinochrome would stimulate the respiration of sperm of *S. purpuratus*. The latter showed that crystalline echinochrome did not increase the motility of the sperm of *A. punctulata*. The paradoxical results of Tyler and Cornman as opposed to Hartmann et al. may be attributed to species difference. However, it is clearly possible that echinochrome does not activate sperm. The non-existence of a sperm-activating function by echinochrome seems to weaken the argument for the existence of a substance neutralizing that activating factor.

Because of these considerations, the concept of a sperm-inhibitor in the seminal fluid seems to be questionable. In the light of parallel experimental results as re-

gards sperm motility and respiratory activity (Hayashi, unpublished), it is concluded that there is no inhibitor of sperm motility in the seminal fluid of *A. punctulata*. This conclusion does not deny the inhibiting effects of hydrogen ions, the influence of which on the increase of the life-span of the sperm has been shown to be insignificant. To restate the conclusion: excluding the hydrogen ion factor, there is no inhibitor of sperm motility in the seminal fluid of *A. punctulata*.

*The seminal fluid factor in its relation to the activating capacity of the sperm*

Various experiments have proved that spermatozoa suspended in seminal fluid retain their capacity to activate eggs longer than sperm cells suspended in sea water (Tables II, III, IV). The factor in the seminal fluid responsible for the effect is not found in the perivisceral fluid, the factor is not the pH of the medium, and the factor is heat-sensitive (Table III). The seminal fluid factor is also non-dialyzable and precipitable with ammonium sulfate (Table IV). On the basis of these results, it may be tentatively stated that the seminal fluid factor is protein. However, the usual protein tests have not been made, so that this conclusion cannot be drawn with any finality, even though the conclusion is strongly supported by positive micro-Kjeldahl analyses indicating protein content in the order of 2.5 mg. protein per cc. of seminal fluid.

The seminal fluid factor, if protein, may act on the sperm cells in several ways. The factor may serve as a source of nutrient for the sperm, it may act on the sperm through the agency of the colloidal osmotic pressure which its presence establishes in the seminal fluid, or it may act through adsorption on the sperm surface. It is necessary to consider these possibilities carefully, if the mechanism of the action of the seminal fluid factor is to be clarified.

The possibility of the seminal fluid factor's acting as a nutrient will be taken up more fully in a later publication on the effect of the seminal fluid on the respiration of sperm. The statement can be made here that these studies indicate that the factor does not act as a nutrient for the sperm. Likewise, the probable protein nature of the factor argues against the idea of nutrition, for the large size of the molecule would prevent its absorption by the sperm. The fact that seminal fluid contains no reducing sugar is further support for the belief that the seminal fluid affords no nutritive elements for the sperm cells.

The question of the effect of colloidal osmotic pressure in prolonging the functional life of the sperm cell is unsettled. Although the further experimental results on the surface activity of seminal fluid substance validate the conclusions drawn, it is admitted that the effects of colloidal osmotic pressure on sperm longevity is still an open question.

The experimental results given in Tables V and VI constitute support for the idea of surface-action of the seminal fluid factor. The data show that both the sperm surface and the seminal fluid factor are surface-active on glass, and they indicate the possible identity of the sperm-surface-substance and the seminal fluid factor.

The foregoing considerations point strongly to the conclusions that the seminal fluid factor is protein and that it is present both in the seminal fluid and on the sperm surface. Since the seminal fluid factor enables the sperm to retain their fertilizing function, it seems logical to infer that the seminal fluid protein plays a part directly in the fertilization process. The data of Tables VIII and IX give support

to this idea. The experiment of Table VIII reveals the fact that, with the same amounts of sperm, a higher percentage of activation of eggs is achieved by sperm in seminal fluid than by sperm in sea water. Since the experiment was so arranged that the insemination tests were made immediately after the mixing of each solution, the possibility that a large number of sperm in the sea water were immobilized seems unlikely. The conclusion most compatible with the results is that the individual spermatozoon in seminal fluid possesses a greater fertilizing capacity than his fellow in sea water. The mere act of dilution in sea water, therefore, seems to have removed a large part of the activating substance from the surfaces of the sperm cells, reducing their individual activating power.

The idea of variation of the activating power of the individual sperm cell was first expressed by Glaser (1915). He diluted sperm serially in sea water, and found that several sperm cells were required to activate one egg cell, even though only one spermatozoon was required to bring about the biparental effect. Lillie (1915) found that when he used the same method of dilution as did Glaser, the fertilizing power of the suspension was far less than an equal concentration of sperm in a suspension diluted in one step. Although these two workers disagreed in their conclusions, their results point to the validity of Glaser's interpretation, which is confirmed in the present study.

Table IX shows the relative fertilizing powers of suspensions in sea water and seminal fluid after they had been aged for 10 hours. If the results are compared to those of Table VIII, it will be seen that the fertilizing capacity of the seminal fluid sperm suspension was not affected by the aging period but that the fertilizing capacity of the sea water suspension was markedly reduced. There are two possible explanations for the enhanced difference in the activating power of the two suspensions, both of which probably contribute to the effect. It is possible that in the 10-hour aging period, large numbers of the sperm cells in the sea water suspension were immobilized, so that they could not penetrate the jelly envelope surrounding each egg. Thus, the number of sperm cells making actual contact with the egg surface was reduced. The final result would be a decreased percentage of activation. The second possibility is the conclusion derived from the analysis of Table VIII, namely, that a substance functional in activation was removed from the sperm surface. During the 10-hour period, this removal presumably continued, so that the activating power of the individual sperm cell was further reduced. Therefore, even if all the spermatozoa remained motile and capable of making contact with the egg, more sperm cells per egg would be required for activation, and the end results would be a decreased percentage of activation. The experiment, therefore, tends to support the idea of an egg-activating substance on the sperm surface, and, also, shows the close relationship between the motile activity and the activating power of the sperm cell.

Many investigators have postulated the existence of a substance on the surface of the sperm and considered that it was protein in nature. Buller (1902), from observations of the sperm of various echinids, reported that the sperm surface was surface-active, not only on glass, but also on air bubbles. Lillie (1913) discovered that in the presence of egg secretions, the male germ cells of *Arbacia* and *Nereis* became agglutinated. He concluded that the surface of the sperm cell was "sticky." The marked similarity of the agglutination phenomenon to an immunological reac-

tion may be taken to be a strong indication for the protein nature of the responsible agent on the surface of the sperm cell.

More direct evidence came from the work of Popa (1927). Using histochemical technique, this worker concluded that the surfaces of *Nereis* and *Arbacia* sperm were covered with a layer of lipo-protein.

Mudd, Mudd, and Keltch (1929) investigated the surface charge of the sperm cells of various echinids. Using the cataphoresis chamber, they reported that the sperm surface was negatively charged. This negativity they found to be increased after agglutination with egg-water. They concluded that their method made possible the detection of substances on the sperm surface.

Henle (1938) and Henle, Henle, and Chambers (1938) found that heat-sensitive antigens existed on the surface of sperm heads. Their work was done with mammalian sperm. Tyler and O'Melveny (1941) obtained rabbit anti-serum by injection of whole sperm of *S. purpuratus* and *L. anamesus*. The anti-serum was found to agglutinate the sperm of these species. These immunological studies again pointed to the protein nature of the sperm-surface-substance.

The evidence cited is not, perhaps, a complete list. The investigations provide enough experimental data, however, to warrant the tentative conclusion that the sperm-surface-substance is protein in nature.

This sperm-surface-substance and the seminal fluid factor may possibly be identical (Tables V and VI). A strong indication of identity could be established if it were shown that the seminal fluid factor alone can activate eggs. Experiments have been started to investigate this possibility, but as yet no conclusive results have been obtained. Comparable work in this direction has not been done. The effect of protein extracts on egg surfaces was investigated by Favilli (1932) and by Hartmann, Schartau, and Wallenfels (1940), while Sampson (1926) reported the activation of eggs by dialysates and filtrates of sperm suspensions. Since her activating factor was dialyzable, and therefore non-protein, it cannot be compared to the seminal fluid factor. In addition, the remarks of Just (1922, 1928, 1929a and b) criticizing the auto-parthenogenesis of Glaser (1914) and Woodward (1921) are equally applicable to the work of Sampson.

Another possible method of establishing the identity of seminal fluid protein and the sperm-surface-substance is to obtain rabbit anti-serum by the injection of seminal fluid. If the anti-serum thus obtained had the power to agglutinate sperm, the results would constitute substantial evidence for the argument that seminal fluid protein and protein on the sperm surface were the same substance. The experiment, however, was not done.

The identity, therefore, is not established, although there is some evidence in this direction (Tables V and VI). Aside from this point, however, there are experimental results throwing light on the origin of and possible relation between the seminal fluid factor and the sperm surface substance. It would be interesting to know whether these substances are secreted by the sperm cells or not, and whether the sperm-surface substance establishes the seminal fluid substance by passing off into the seminal fluid, or whether the seminal fluid substance establishes the sperm-surface substance by adsorption on the sperm surface.

Numerous investigators have reported that sea-urchin sperm give off a substance into the surrounding sea water (Lillie, 1914 and 1915; Southwick, 1939; Hartmann,

1940; Hartmann, Schartau, and Wallenfels, 1940) and that this substance showed protein characteristics (Frank, 1939; Tyler and O'Melveny, 1941). All investigators agreed that the substance showed the properties of "antifertilizin" or the power to "bind" the agglutinin of egg-water so that the agglutinating effect on sperm was reduced.

The question here posed is: does this substance from the sperm cells have the properties of the seminal fluid substance? The point was tested by an experiment in which a sea water suspension of sperm was allowed to stand for several hours, the sperm cells removed by centrifugation, and a fresh sample of sperm suspended in the medium. The results were negative. This medium was not effective in prolonging the fertilizing capacity of sperm, and therefore did not have the properties of the seminal fluid substance.

There is the converse question: Does the seminal fluid substance have the antifertilizin property of the substance coming off the sperm cell? Again, the results were negative (Table X).

The substance coming off the sperm cell does not have the properties of the seminal fluid substance. The results of these experiments indicate, therefore, that a sperm substance does not establish the seminal fluid substance, so that the seminal fluid factor does not have its origin in the sperm cell. The same negative answer as to the origin of the sperm-surface substance cannot be given.

However, the fact that the substance coming off the sperm surface has different properties from the seminal fluid substance signifies nothing regarding the properties of the sperm substance while on the sperm surface. This substance on the surface of the sperm cell is surface-active on glass, as is the seminal fluid substance (Tables V and VI). The seminal fluid factor also enables the sperm cell to maintain its fertilizing capacity longer (Tables II, III, IV) and seems to enhance the fertilizing power of the individual spermatozoön. In addition, the seminal fluid factor affects the surface of the sperm so that the time of agglutination, or the reaction with agglutinin, is increased (Table XI).

These facts point to a tentative explanation of the relation between the seminal fluid factor and the sperm-surface substance. It is possible that a protein substance, originally present in the seminal fluid, is adsorbed on the surface of the sperm cell, thus influencing the fertilizing power of the sperm cell, as well as rendering the surface of the sperm cell surface-active. By this adsorption also, the sperm surface is rendered capable of greater reactivity with fertilizin. The subsequent loss of this substance from the sperm cell results in the loss of fertilizing power and the presence of antifertilizin in the sperm medium. The antifertilizin would be, according to this scheme, a substance changed in certain properties from the original seminal fluid substance.

#### SUMMARY

1. A factor is present in the seminal fluid of *Arbacia punctulata* which prolongs the fertilizing capacity of the sperm cells of the same species.

2. The factor, which is not found in the coelomic (perivisceral) fluid, is heat-sensitive, precipitated by saturation with ammonium sulfate, non-dialyzable, and surface-active on glass. Since micro-Kjeldahl analysis of the seminal fluid gives positive results corresponding to 2.5 mg. protein per cc. of seminal fluid, it is tentatively suggested that the factor is protein.

3. Seminal fluid has a pH range of 7.6 to 7.9, its osmotic pressure is approximately 10 per cent lower than sea water, and its content of reducing sugar is negligible.

4. In equivalent concentration and immediately after suspension the fertilizing capacity of the individual spermatozoön is greater in seminal fluid than in sea water.

5. Seminal fluid does not contain antifertilizin since it does not neutralize the agglutinating action of egg-water; indeed, this action is intensified.

6. A tentative mechanism, based on the adsorption of a fertilizing substance and its removal from the surface of the sperm cell, is suggested to explain the experimental results. It is proposed that the seminal fluid factor is this fertilizing substance before adsorption and while on the surface of the sperm; it becomes changed upon removal from the sperm surface.

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# HETEROCINETA PHORONOPSIDIS SP. NOV., A CILIATE FROM THE TENTACLES OF PHORONOPSIS VIRIDIS HILTON

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## INTRODUCTION

The infestation of the tentacles of *Phoronopsis viridis* Hilton by a small ciliate of the family Ancistrocomidae Chatton and Lwoff<sup>1</sup> (order Holotricha, suborder Thigmotricha) was called to my attention by Professor Harold Kirby. A preliminary study of this ciliate, from slides prepared in his laboratory from material collected in Bodega Bay, California, in November, 1943, disclosed that on the basis of the organization of the ciliary system it appeared to be most closely related to species of the genus *Heterocineta* Mavrodiadi, ectoparasitic on fresh water mussels, prosobranchs, and pulmonates (Jarocki; 1934, 1935).

In June, 1945, I collected additional material of *Phoronopsis viridis*<sup>2</sup> in an intertidal mud flat in Tomales Bay. From observations of the living ciliates it was determined that this new species, which will be described herein as *Heterocineta phoronopsidis* sp. nov., differs fundamentally from other species of *Heterocineta* in having a groove-like depression originating on the left side of the body near the anterior end and extending posteriorly along the dorsal surface close to the left margin. I have studied a species of *Heterocineta* ectoparasitic on *Physa cooperi* Tryon from a locality near Mt. Eden, California, which agrees perfectly with the description of *Heterocineta janickii* given by Jarocki (1934). This ciliate, like *H. phoronopsidis*, has eight ciliary rows, but these are restricted to a more narrow area on the ventral surface. There is no dorso-lateral groove in *H. janickii*. In none of Jarocki's descriptions of ciliates of the genus *Heterocineta*, which apparently were based to a large extent upon living material, is there any mention of such a groove.

## TECHNIQUE

For observation of the living ciliates the tentacles of *Phoronopsis viridis* were detached from the rest of the body by means of forceps and comminuted in a drop of sea-water on a slide. Fixation of the organisms for permanent preparations was accomplished by preparing smears in this manner on coverglasses and dropping them onto the surface of the fixative in a Petri dish. For a study of the general

<sup>1</sup> Chatton and Lwoff (1939) proposed the family Ancistrocomidae to include those ciliates formerly assigned to the family Hypocomidae Bütschli which differed from the type genus of the latter (*Hypocoma* Gruber) in having the suckorial tentacle disposed terminally rather than subterminally and the ciliary rows arranged singly rather than in pairs.

<sup>2</sup> Professor W. A. Hilton of Pomona College has kindly identified the phoronid species from Tomales Bay as *Phoronopsis viridis* Hilton (1930). It should here be noted, however, that no systematic revision of the phoronids from the Pacific Coast has been given in the literature and it is not impossible that *P. viridis* will later be shown to be identical with one of the species described earlier.

morphology, staining with iron hematoxylin gave good results on material fixed in Schaudinn's fluid. Differentiation of the ciliary system by impregnation with activated protein silver (protargol) was successful following fixation in Hollande's cupric-picro-formol mixture and Schaudinn's fluid, but this method was no more satisfactory than staining with iron hematoxylin. The Feulgen nuclear reaction was used after fixation in Schaudinn's fluid and a saturated aqueous solution of mercuric chloride with 5 per cent of glacial acetic acid.

#### HETEROCINETA PHORONOPSIDIS sp. nov.

The body is elongated, asymmetrical, and flattened dorso-ventrally. Twenty living individuals taken at random ranged in length from  $26\ \mu$  to  $37\ \mu$ , in width from  $11\ \mu$  to  $16\ \mu$ , and in thickness from  $6.5\ \mu$  to  $11\ \mu$ , averaging about  $29\ \mu$  by  $14\ \mu$  by  $8\ \mu$ . As seen in dorsal view (Fig. 1A) the left side of the ciliate is conspicuously rounded, while the right side is by comparison very little curved. The body is usually widest at a point a short distance behind the middle and is rounded posteriorly. The attenuated anterior end is deflected toward the left, truncate at the tip, and bent ventrally. The reduced ciliary system, to be described presently, is disposed in a shallow concavity occupying the anterior four-fifths of the ventral surface (Fig. 1B); the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex.

A contractile suctorial tentacle enables the ciliate to attach itself to epithelial cells of the tentacles of the host and to feed upon their contents. When fully extended the suctorial tentacle of *Heterocineta phoronopsidis* is about  $4\ \mu$  in length and  $1.5\ \mu$  in diameter; it is contracted as soon as the ciliate is dissociated from the host and is seldom preserved in an extended condition in fixed individuals except those which have been fixed in a position of attachment to the host.

The internal tubular canal (Fig. 1, c) continuous with the suctorial tentacle is about  $1.5\ \mu$  in diameter in its anterior portion, which is directed dorsally, and becomes abruptly narrower in its posterior portion, which is directed ventrally and obliquely to the right. In some living specimens and in suitable preparations stained with iron hematoxylin the canal can be traced along the right side of the body to a point a short distance posterior to the macronucleus.

The cilia of *Heterocineta phoronopsidis* are about  $5\ \mu$  in length and markedly thigmotactic. They are disposed in eight longitudinal rows limited to the shallow concavity on the ventral surface (Fig. 1C). All eight rows originate near the base of the suctorial tentacle. Each of the first five rows from the right margin is about three-fifths the length of the body. The fourth and fifth rows are as a rule practically straight, while the outer three are appreciably curved. The remaining three rows become progressively longer and inflexed in such a way that they end one behind the other near the mid-line. The eighth and longest row terminates at a point about four-fifths the distance from the anterior end of the body to the posterior end. The cilia of the anterior part of the thigmotactic system move rather actively, those of the posterior part sluggishly.

The shallow groove-like depression which distinguishes *Heterocineta phoronopsidis* from other species of *Heterocineta* has its inception on the left side of the body near the anterior end and is about four-fifths the length of the body (Fig. 1A, g). As it extends posteriorly it comes to lie on the dorsal surface along the left margin.

The groove is visible only in living individuals. There are no traces of ciliature at any point along its course. Staining with iron hematoxylin and impregnation with protein silver fail to bring out any basal granules in the region occupied by the groove.

The cytoplasm is colorless and contains a number of small refractile granules in addition to food inclusions. The refractile granules (Fig. 1A, cg) are apparently lipid droplets, as they are dissolved out by toluol used for clearing following staining. At least one large food-vacuole and usually several smaller ones are present near the posterior end of the body (Fig. 1, fv). The contents of the food-vacuoles

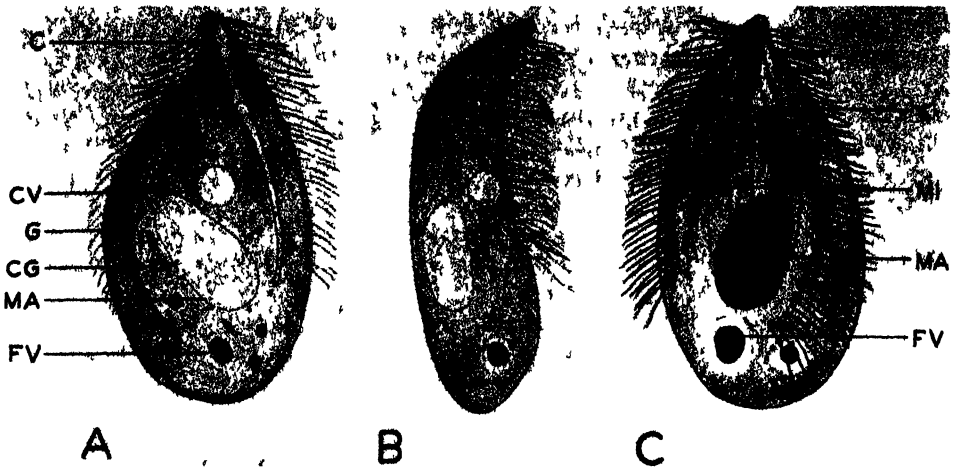


FIGURE 1. *Heterocineta phoronopsidis* sp. nov.

A Dorsal aspect, from life; B, lateral aspect from right side, from life; C ventral aspect. Schaudinn's fixative-iron hematoxylin. Drawn with aid of camera lucida  $\times 1940$ .

c = internal tubular canal, cg = cytoplasmic granule, cv = contractile vacuole, fv = food vacuole, g = dorso-lateral groove, ma = macronucleus, mi = micronucleus

are seen to consist mainly of ingested nuclei or fragments of nuclei from the epithelial cells of the tentacles of the host

The contractile vacuole (Fig. 1, cv) lies near the middle of the body and opens to the exterior on the ciliated ventral surface. I have not distinguished a permanent opening in the pellicle.

The oval or rod-shaped macronucleus (Fig. 1, ma) is placed dorsally near the center of the body, its longitudinal axis lying obliquely to the longitudinal axis of the body. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen nuclear reaction on the macronucleus ranged in length from  $5.25\ \mu$  to  $7.5\ \mu$  and in width from  $3\ \mu$  to  $4.5\ \mu$ .

The fusiform, rod-shaped, or crescentic micronucleus (Fig. 1C, mi) is situated anterior to the macronucleus. It is very difficult to distinguish in living specimens and is stained only weakly by iron hematoxylin and the Feulgen nuclear reaction. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction

the micronucleus ranged in length from  $1.5\ \mu$  to  $2.25\ \mu$  and in width from  $0.75\ \mu$  to  $1.2\ \mu$ .

When attached to the tentacles of the host *Heterocineteta phoronopsidis* is almost immobile, exhibiting only a passive vibratory motion due to the energetic movement of the epithelial cilia. When dissociated from the host the ciliate swims slowly, usually rotating on its longitudinal axis and tracing wide arcs with its attenuated anterior end.

*Heterocineteta phoronopsidis* sp. nov.

Diagnosis: Length  $26\ \mu$ – $37\ \mu$ , average about  $29\ \mu$ ; width  $11\ \mu$ – $16\ \mu$ , average about  $14\ \mu$ ; thickness  $6.5\ \mu$ – $11\ \mu$ , average about  $8\ \mu$ . The anterior end is attenuated, bent ventrally, and provided with a contractile suctorial tentacle continuous with an internal tubular canal. The ciliary rows are eight in number and originate near the base of the suctorial tentacle. The first five rows from the right are about three-fifths the length of the body, while the remaining three rows become progressively longer and are inflexed in such a way that they end one behind the other near the mid-line. A groove-like depression, without any trace of ciliature, extends from the anterior end of the body posteriorly along the dorsal surface close to the left margin. Ectoparasitic on the tentacles of *Phoronopsis viridis* Hilton (Tomales Bay, California). Syntypes are in the collection of the author.

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## ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1945

### *The role of bacteria in the excystment of the ciliate Didinium nasutum.* C. D. Beers.

Resting cysts were obtained by the completion-culture method, viz., by preparing small cultures in spring water with paramecia as food. Most of the didinia in such cultures encysted upon exhaustion of the food supply. Such cysts never became active spontaneously, nor were they bacteria-free.

Distilled water, sugars, salts of plant acids, pH changes, and metabolites of *Paramecium* were ineffective in inducing excystment.

Timothy and lettuce infusions, and peptone and yeast-extract solutions, induced 78-94 per cent excystment within 9-12 hours at 28° C. The tentative conclusion that these substances were effective excystment-inducing agents *per se* was soon negated by the observation that at the time of excystment, bacteria (introduced with the cysts) were always flourishing in the media, which had been originally sterile. To test more adequately the effect of bacteria, these same four media were inoculated with wild bacteria from *Paramecium* cultures and incubated 18-24 hours. The bacterized media when tested on cysts induced 89-95 per cent excystment within 3-4 hours at 28° C. and thus produced a distinct acceleration effect.

The special effectiveness of bacterized peptone suggested an examination of the role of amino acids in excystment. Nine such acids were tested, singly and in mixtures, in buffered solution, but none yielded an accelerative effect. Only those acids (e.g., histidine, arginine, proline, methionine) and mixtures which supported bacterial growth induced excystment, and then only when bacteria were flourishing, i.e., after 9-12 hours. Acid mixtures previously inoculated with bacteria produced the usual accelerative effect. Hydrolyzed peptone behaved similarly.

Boiling the bacterized acid mixtures, or peptone solutions or hydrolyzates destroyed their effectiveness, which, however, could be restored by inoculation with bacteria.

The results indicate that excystment in *Didinium* is induced through the agency of bacterial action. Further studies are in progress to identify the effective bacteria, and to ascertain the chemical nature of the substances responsible for excystment.

### *Cytological studies in Culex.* C. A. Berger and Sister Mary Grell.

Cells in the hind-gut of *Culex* ( $2n = 6$ ) are diploid at the beginning of larval life and are highly polyploid at pupation. This polyploid condition arises by repeated chromosome reduplication within the resting nucleus. During metamorphosis these cells undergo mitotic division. The first division of a 16-ploid cell is described. Unique cytological features of this division are as follows. There are six groups of chromosomes each composed of eight sister chromosomes. Homologous groups are paired, relationally coiled and apparently have their spindle attachment regions fused. In early prophase the association of sister chromosomes is so close that the eight appear as one. As prophase contraction proceeds the eight sister chromonemata become evident and are seen to be relationally coiled in two's, in pairs of two's, etc. The spindle attachment region undergoes successive division in late prophase. At metaphase 48 chromosomes can be counted. Anaphase separation is regular and homologous or sister chromosomes pair as they move to the poles. This work can be interpreted as favoring the first part of Darlington's hypothesis, that chromosomes are attracted in pairs only, but gives no support to the second part of the hypothesis, that pairs of pairs repel.

### *Accelerating metamorphosis in the tunicate, Styela partita.* Lloyd M. Bertholf.

C. Grave discovered that metamorphosis in tunicate larvae can be hastened by dozens of different substances, from complicated extracts of tunicate and vertebrate tissues down to simple salts of several heavy metals, added to sea-water. He concluded that such acceleration

is caused by a poisoning of the larval action-system, so that the adult action-system takes over sooner than normally, and that the chief agent in this poisoning is copper.

To ascertain how specific the need for copper is, an effort was made to hasten metamorphosis by various substances in which copper is absent. Isotonic solutions of NaCl alone or in combination with other salts and with lactose and sucrose were first used. All these solutions brought about metamorphosis much sooner than in the controls, provided the larvae were about 4 hours old or older; if younger, the animals usually died before metamorphosis or shortly afterward.

It is possible, however, that the salts used contained a threshold amount of copper and other heavy metals as impurities. Hence distilled water alone was next used. This killed the animals after a few minutes of continuous exposure, but if larvae of about 2 hours or older were immersed in distilled water for only  $\frac{1}{2}$  to 2 minutes and then transferred to normal sea-water, metamorphosis was much hastened, and no deleterious effects resulted.

It seems, then, that the effect of copper is not specific, but that similar effects can be produced by other means, including the physical shock of a large change in osmotic pressure.

#### *Oxidation-reduction studies on Penicillium notatum and other organisms.* Matilda Moldenhauer Brooks.

Redox potential and pH measurements by means of the Coleman electrometer were made of the media in which *Penicillium notatum* and several other organisms were grown. Daily readings were taken for a period of several weeks. *Penicillium* was grown in corn steep medium. *Aspergillus flavus*, *Mycoderma*, *Torula utilis* and *Sacchromyces cerevisiae* were grown in modifications of Czapek-Doz media. Sterile conditions were maintained.

It was found that rH values ( $= 2 \text{ pH} + E_h/.03$ ) for *Penicillium* were 8.4 to 8.7. For purified penicillin (100,000 Oxford units) the rH was 8.7. For other organisms it was either higher or lower. In the case of *Penicillium*, the  $E_h$  value became very negative ( $-0.25$ ) and the pH, alkaline (8.5). No other organism studied had these characteristics.

When flasks were tightly stoppered, the rH values were similar to those obtaining in cultures to which KCN had been added. Growth was hindered when aerobes were used and not affected in the case of facultative anaerobes.

It is suggested that the therapeutic action of penicillin and related organisms depends upon the balance between  $E_h$  and pH in the blood, which these organisms produce. This factor makes it incompatible for such organisms as *Staphylococcus aureus*, for example, to exist.

#### *Organization of the giant nerve fiber system in Neanthes virens.* Theodore H. Bullock.

The presence of giant nerve fibers in certain polychaete annelids has been known on the basis of anatomical studies, but their function and functional organization have not been investigated. The group is especially suitable for such studies since its members present a great diversity of neural development; giant fibers are present in varying pattern in many species, absent in others; the group is large, and favorable species for laboratory study are common. A survey of the functional anatomy of the giant system in representative forms has been undertaken to the end of adding perspective to our picture of the evolution of the nervous system and with the hope of finding material for special studies of nervous physiology. The electrical signs of nervous activity were used as a tool for revealing the functional anatomy.

The present report will be confined to *Neanthes virens* (*Nereis virens*). When the nerve cord is directly stimulated by single shocks there is recorded from the nerve cord or from the mid-ventral line of the intact animal, in any other part of the worm, a pattern of large spikes, several orders of magnitude higher in voltage than the action currents representing spontaneous activity of the small fibers of the nerve cord. These large spikes have the properties of single fiber action currents. The first is the largest, has the lowest threshold, fatigues the slowest, and arrives at a time representing a minimum conduction rate (assuming no delays) of 5 meters/sec. Unlike the others it is not all or none, but all or half or none; two independent units are present conducting at just the same rate but separable by threshold and fatigue. The second spike is intermediate in height, threshold, fatigue, and rate (4.5 m/s) between each half of the first spike and the later ones. A small third spike at 2.5 m/s may be alone or followed by another

like itself. This pattern is constant from specimen to specimen and may be regarded as characteristic of the species. One can expect anatomically at least four giant fibers or conducting units: a pair larger than the rest but identical in average diameter; a single unit, next in size; and one or two small but still "giant" units. This corresponds precisely with the known anatomy, there being a pair of large lateral fibers, a smaller median unpaired fiber, and a pair of still smaller medially placed fibers. The present technic can assure certain relations difficult to establish histologically. There is no anastomosis between the lateral fibers such as occurs in *Lumbricus*; the fibers are all independent conducting units, none being a necessary efferent or afferent connection of another; all the fibers are unpolarized, conducting equally well in both directions (although segmental macrosynapses like those in *Lumbricus* have been described). The sensory connections of each fiber can be inferred from responses to mechanical stimuli. The giants can each be fired through sense organs by local stimulation of the skin (a light tap or dropping water) within certain segmental levels; the head is not necessary. The median fiber (second spike with direct electrical stimulation) is fired by stimuli in the anterior quarter, approximately; the smallest, slowest giants by stimuli in the posterior three quarters and a region of overlap of a few segments occurs. The fast lateral giants can be fired from any level but require stronger stimuli (water dropping from a few cms. higher for example). The evidence suggests a function as mediators of startle responses to three classes of stimuli—weaker anterior, weaker posterior, and stronger at any level (differences in threshold in different levels exist for each fiber within this scheme). The two laterals usually fire together but in certain cases they can be separated.

The plan in general is very like that in *Lumbricus* although the two belong to different classes and many polychaetes with just as close a relation have no or very differently organized giant systems.

#### *The displacement of terns by gulls at the Weepeeket Islands.* Sears Crowell.

The changes in population at the colony of Common and Roseate Terns at the Weepeeket Islands are described for a period of twenty years. The colony attained, by 1931, a population of 3500 adult terns. During the past ten years this colony of terns has gradually been replaced by breeding Herring Gulls. The terns are probably incapable of successful reproductive activity if gulls are near, even though the latter do no direct injury to the terns.

The members of the Weepeeket colony have been redistributed among other colonies of southern Massachusetts as shown by recoveries of banded birds.

Conditions favor a recolonization by the terns if the gulls are evicted or abandon the islands.

#### *The influence of drugs on heat narcosis.* A. Froehlich.

When the temperature of the surrounding water is slowly raised aquatic animals, such as crustaceae, fishes, tadpoles and frogs, show complete loss of voluntary and reflex muscular activity at a "critical point" of temperature which is characteristic for each species. This condition is reversible; transference into cool water causes the animals to recover promptly. "Heat narcosis" resembles narcosis brought about by drugs (alcohol, ether, etc.) in every way, except that where the former increases oxygen consumption, the drugs diminish oxygen consumption.

For reasons too numerous to mention here, I decided to investigate the influence of theophylline (as theophylline natrio-aceticum) on the "critical point" of heat narcosis. The experiments were performed on *Fundulus heteroclitus* at the M.B.L. in Woods Hole during the summer months of 1944 and 1945 and at the May Institute for Medical Research, Cincinnati, Ohio, on field frogs and tadpoles during the winter and spring of 1944-1945.

Theophylline given subcutaneously or intramuscularly in doses which had no visible effect on the behavior of the experimental animals produced a considerable lowering of the "critical point" in heat narcosis. The same effect was obtained if the animals were placed in a weak solution of theophylline.

Theophyllinized animals died much sooner than did controls if access to air was restricted. The water in which such animals died showed far greater acidity due to accumulation of CO<sub>2</sub>.

Asphyxiation alone produced a lowering of the "critical point" similar to that obtained with theophylline.

Methylene blue (intramuscularly to *Fundulus*) produced effects on the "critical point," susceptibility to asphyxiation and acidity of the water which were similar to those obtained with theophylline.

In the experiments with theophylline as well as in those with methylene blue, previous conditioning in a 1:100,000 solution of quinine sulfate counteracted to a greater or a lesser degree the expected lowering of the "critical point."

It can be concluded that the action of theophylline and methylene blue on these experimental animals is, in part at least, to increase the demand for oxygen, and that quinine reverses this action by decreasing respiratory metabolism.

As I had previously found (with E. Zak) that an important part of the action of theophylline consists in increasing tissue permeability, I feel justified now in assuming that this phenomenon is caused by a condition of hypoxemia and acidosis (local asphyxia) in the tissues.

### *Reactions of oyster (Ostrea virginica) to free chlorine.* Paul S. Galtsoff.

By measuring the rate of flow of water through the gills and by recording the shell movements it was possible to demonstrate that both the pumping mechanism of the oyster and its adductor muscle are very sensitive to free chlorine. In many oysters the first treatment with the concentrations as low as 0.01 or 0.02 p.p. million causes complete cessation of current and closure of shells, although there are specimens in which complete cessation of pumping and closing of shells takes place only in the concentrations approaching 0.5 p.p.m. Repeated treatments develop increased tolerance and pumping may be resumed at the concentrations much stronger than those which produced strong initial effect. Pumping, however, is not maintained at the concentrations of one p.p.m. or greater.

Variation in the sensitivity and development of tolerance are apparently associated with the secretion of mucus which provides protective coating for tentacles, mantle, and gills. Observations with a strobotac show that lateral cilia of the excised gill filaments continue to beat even at the concentration of 3 p.p.m. The cessation of pumping activity of an intact organism is due, therefore, not to the failure of the lateral cilia, but to the reaction of the regulatory mechanism of the pallium, which prevents the entrance of water to the gills, and to a certain extent to the disturbance of the rhythm of ciliary motion over the entire ciliated surface of the demibranches.

The presence of free Cl in water may materially impede the purification of oysters. It is therefore necessary that water, sterilized by chlorination and used in a process of purification, contains no residual Cl.

### *Development of granule-free fractions of Arbacia eggs.* Ethel Browne Harvey.

A granule-free fraction of the *Arbacia punctulata* egg is obtained by breaking the egg with centrifugal force into two halves, and then breaking the lighter (white) half into two quarters, one of which contains all the remaining granules; and the other, the "clear quarter," is free of all granules visible in the living egg. This clear quarter contains the oil, nucleus, and most of the matrix or ground substance, but no mitochondria, yolk, or pigment. When fertilized, this clear quarter in many cases throws off a fertilization membrane, cleaves quite regularly, forms a perfect blastula and gastrula and pluteus. This pluteus may be quite normal with gut and skeleton, and later develops pigment spots, but is much clearer and less granular than that from the white half. It is approximately half the size of the pluteus from the white half and quarter the size of the pluteus from the whole egg. There is a considerable delay throughout development beginning with first cleavage, in spite of the fact that two nuclei (♂ and ♀) are present with a small amount of cytoplasm. The visible granules of the egg are therefore not necessary for development. The important substance in the cytoplasm is the ground substance or matrix, which is optically empty in the living state.

### *In vivo and in vitro glycogen utilization in the avian nematode Ascaridia galli.* W. Malcolm Reid.

Glycogen constitutes one-third or more of the dry weight of many parasitic nematodes and flatworms. Extensive *in vitro* experiments upon glycogen utilization have been carried out by different investigators chiefly upon mammalian nematodes, cestodes, and trematodes. Von

Brand with *Ascaris lumbricoides* showed that 45 per cent of the glycogen reserve was utilized by females during 48 hours. Recent experiments upon fowl nematodes and cestodes have shown a much higher rate of glycogen utilization when the host had been starved for a short time. In a typical experiment with *Ascaridia galli*, 75 per cent of the glycogen reserve was utilized in 48 hours by female worms. With the fowl cestode, *Raillietina cesticillus*, this reserve was depleted even more rapidly, 94 per cent of the glycogen being utilized in 24 hours.

Until a study using simultaneously *in vivo* and *in vitro* methods upon the same parasite has been completed, a comparison of the results of such experiments can have little meaning. Furthermore, such a study would serve as a check upon the earlier *in vitro* experiments which need re-examination now that improvements in technique have brought some of these results under question.

Glycogen determinations were made upon three groups of *A. galli*. Group I were controls and consisted of worms which were removed from the host after a normal feeding period. Group II worms were starved within the host for 48 hours before glycogen determinations were made. Group III consisted of parasites removed from the same hosts used for Group I, but these parasites were starved anaerobically for 48 hours at  $41.5^{\circ} \pm 1^{\circ}$  C. in one per cent saline using the same *in vitro* methods that were used on mammalian forms. Separate determinations were made on both males and females since sex differences in glycogen content were known to exist. The mean glycogen content for approximately ten samples for each group expressed in per cent of the wet weight of the worms is as follows: Group I females, 4.66; Group II females, 1.16; Group III females, 1.01; Group I males, 3.81; Group II males, 0.43; and Group III males, 0.26. The similarity in the rate of glycogen utilization with both males and females under the two conditions probably indicates that the *in vitro* methods used by early investigators reflect reliable information about normal glycogen metabolism within the host. Comparison between the glycogen utilization in the avian *A. galli* with the mammalian *A. lumbricoides* indicates that the much higher utilization rate in *A. galli* is real and not due to differences in technique.

#### *Balanced centerwell solutions for manometric experimentation with cyanide.*

W. A. Robbie.

It has been demonstrated, both experimentally and theoretically, that the potassium cyanide-potassium hydroxide absorption solutions recommended by Krebs (1935, *Biochem. Journ.*, 29: 1620) are not in hydrogen cyanide equilibrium with the experimental fluids for which they were designed. It is possible, however, to prepare, on the basis of experimental determinations, potassium cyanide-potassium hydroxide mixtures which will absorb carbon dioxide and maintain hydrogen cyanide equilibria with cyanide solutions of 0.011 M or less. The hydrogen cyanide tension of calcium cyanide solutions saturated with calcium hydroxide varies only with the concentration of the calcium cyanide and the temperature. This type of centerwell mixture will absorb carbon dioxide effectively and maintain equilibrium with hydrogen cyanide solutions up to 0.01 M.

#### *Studies of the muscle twitch recorded by electronic methods.\** Alexander Sandow.

Piezoelectric, cathode-ray oscillographic methods have been devised for recording the various mechanical changes of the isometric twitch of skeletal muscles. To register the latency relaxation, LR (the minute precontractile elongation of a stimulated muscle during the latter half of the latent period), the apparatus is used, in effect, as an electronic lever which converts the LR into a  $500,000 \times$  magnified deflection on the cathode-ray screen. The piezoelectric pulse corresponding to the main contraction and relaxation periods is electronically differentiated and thus at each instant the cathode-ray deflection for this record is proportional to the rate of tension change in the course of the twitch.

These methods have been used to study the effect of maximal tetani of lengths from  $\frac{1}{8}$  to 10 sec. on the mechanical features of the twitch of the frog sartorius. The results prove that the separate processes that underlie the LR, the use of tension, and the post-contractile relaxation, are each uniquely affected by the tetanic activity. E.g., a 2 sec. tetanus causes a 10 per

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cent increase in the maximum rate of tension rise in a twitch, but a 40-60 per cent increase in the maximum rate of relaxation. The great lability of the relaxation process associated with the new chemical environment induced by the activity is specially significant in indicating that relaxation is not passive but is chemically driven.

The LR shows certain temporal features like those of Brown's alpha-process, thus indicating that it is an external mechanical sign of the alpha-process. Detailed analysis of the effect of activity and of pH on the LR, especially in reference to the duration of the latent period, suggests that the latent period is an interval during which myosin-ATPase is splitting ATP, and leads to the inference, now being subject to further test, that the LR corresponds to the formation of an enzyme-substrate complex between myosin and ATP which provides a mechanism for directly energizing and activating the myosin for contraction.

*Experimentally induced tumors in an insect.* Berta Scharrer.

In *Leucophaea maderae*, a large Orthopteran, the recurrent nerve was cut at various levels. This nerve, which belongs to the stomatogastric nervous system, innervates the anterior portion of the alimentary canal as well as the salivary glands and their reservoir. Within ten days to several months after the operations tumors developed in organs innervated by the recurrent nerve. Frequent sites of tumorous growth were the anterior portion of the mid-gut and the salivary reservoir. In the fore-gut and in the salivary glands well developed tumors were relatively rare. Several types of control operations, such as allatectomy and castration in which the recurrent nerve had remained intact, did not cause the development of tumors. Some of the tumors obtained after the cutting of the recurrent nerve attained considerable sizes. Histologically they consist of layers of cells which show various degrees of abnormality. In advanced stages part of the cells break down into a debris of brown color. About 300 specimens, nymphs as well as male and female adults, with experimental tumors, were studied.

*The origin of neurosecretory granules from basophil constituents of the nerve cells in fishes.* Ernest Scharrer.

Neurosecretory granules do not appear to be formed in association with the Golgi apparatus or the mitochondria, but with the basophil constituents of the secreting nerve cells. Three modes of origin of the granules have been observed. In the preoptic nucleus of most fishes the granules originate in association with the peripherally located Nissl bodies. The latter diminish to the extent to which the acidophil neurosecretory granules increase. In a second type found in the preoptic nucleus of *Ameiurus*, *Noturus*, *Centropomus*, and others the nuclei of the secreting nerve cells show invaginations. These are filled with basophil cytoplasm which may contain acidophil granules. In a third type which is characteristic of the nucleus lateralis tuberosus of catfishes, the acidophil granules originate within the nuclei of the cells, apparently at the expense of the nuclear chromatin. All three types may occur in the preoptic nucleus of *Centropomus*.

*Evidence of a metabolic effect by potassium in lowering the injury potential of invertebrate nerve.\** Abraham M. Shanes.

The action of potassium on the injury potential of spider and blue crab nerve has been studied over a concentration range of one to 530 mM. When the magnitude of these potentials is plotted against the logarithm of potassium concentration, the relative effectiveness of low potassium concentrations in lowering the potential is found to be  $\frac{1}{2}$  that of concentrations above 30 to 40 mM. The data may be replotted on a log-log graph on the assumption that potassium is inactivating an enzyme, the active form of which is proportional to the resting potential. Two straight lines intersecting at 40 mM fit the data very well, the slope at lower concentrations being about  $\frac{1}{2}$  and at higher concentrations about one. This graph is like one which has been obtained for the effect of urethane on oxygen consumption in yeast and *Arbacia*; in this case the inhibitor is believed to act on two processes. The same interpretation may be applied to potassium.

\* Aided in part by a grant from the American Academy of Arts and Sciences.

The similarity of potassium to an actual inhibitor is even stronger if consideration is given to the effect on activity. Only at a concentration corresponding to almost complete cessation of the process affected at low concentration does activity appreciably and suddenly decrease. Thus, in crab nerve, conduction ceases between 37 and 42 mM. Potassium and excitability is unaffected up to 37 mM.

The effect of low potassium concentrations is definitely correlated with the simultaneous inhibition of an aerobic metabolic process which supports the injury potential. In concentrations of 10 to 30 mM potassium eliminates  $\frac{2}{3}$  of this process—values corresponding closely to those obtained previously in frog nerve.

*Physical-chemical studies on chromosomal nucleoproteins.\** Kurt G. Stern.

The object of this research is to determine the size and shape of desoxyribosenucleoproteins, isolated from cell nuclei, with the aid of such quantitative methods as ultracentrifugation, diffusion, electrophoresis, viscosity, x-ray diffraction, and similar techniques. In this cooperative study, S. Davis, P. Macaluso, S. C. Shen, and I. Fankuchen are collaborating with the writer.

Thus far, the desoxyribosenucleoproteins from the nuclei of chicken red blood cells and from calf thymus gland have been studied. Measurements in the analytical ultracentrifuge, in the diffusion apparatus, and in Ostwald viscometers, performed on solutions of these purified nucleoproteins in one M. NaCl, indicate a molecular weight of the order of two to three million and axial ratios varying from 35:1 to 100:1. The discrepancy of the results obtained with independent techniques casts considerable doubt on the suitability of this solvent, proposed by Mirsky and Pollister, with regard to the native state of the nucleoproteins. It appears that these conjugated proteins are appreciably dissociated in M. NaCl-solution. According to preliminary experiments, one M. glycine appears to be a solvent better suited for physical-chemical studies on these macromolecules.

The theory that these desoxyribosenucleoproteins are capable of assuming a more or less *helical shape* in solution as a function of the nature and ionic strength of the solvent, is advanced as a working hypothesis. Thus it is assumed that these molecules reflect in their configuration, on a molecular scale, the coiling and uncoiling of the chromosomes of which they represent important constituents. Plastic models, constructed in accordance with this hypothesis, were demonstrated at the Seminar.

*Action of quitenine on the livers of tautog and toadfish.* Charles H. Taft.

When quinine is treated with potassium permanganate the vinyl group is oxidized to a carboxyl group yielding quitenine.

It has been shown (Dauber, M., 1920; *Zeit. für Expt. Path. u. Therapie*, 21: 311) that quitenine had a damaging action on kidney tubules. Taft and Place (1944; *Texas Reports on Biol. and Med.*, 2: 61) showed that quitenine was more injurious to the kidneys of a glomerular fish than to the kidneys of an aglomerular fish.

Quitenine dihydrochloride in a 0.25 molar solution was injected subcutaneously into the side of the fish. The doses used were 1, 2, and 4 mM/Kg. Fish were killed by a blow on the head after varying intervals of time. The livers were placed in Bouin solution. Sections were cut 6  $\mu$  thick.

On gross examination a few tautog livers were abnormally soft. Gall bladder was a greenish blue in all cases. In the toadfish the liver was soft in a few cases. Color of gall bladder ranged from white through pale pink, orange, yellow green to green. Variation in color is probably due to variation in amount of bile pigment production or to oxidation of bile pigment. The von Kupffer cells were undamaged as were pancreatic cells of hepatopancreas.

Microscopic examination of toadfish liver shows fatty metamorphosis and some parenchymatous degeneration. Microscopic examination of the tautog liver showed fatty metamorphosis, albuminous degeneration, hydropic degeneration, and parenchymatous degeneration.

Quitenine is more damaging to the liver of the tautog than to the liver of the toadfish. The damage to the livers is not as severe as it was in the kidneys.

\* This work was made possible by a grant of The Carrie S. Scheuer Foundation of New York.

*Differences in sensitivity, hatchability curves, and cytological effects between Habrobracon eggs x-rayed in first meiotic prophase and metaphase.* Anna R. Whiting.

Unlaid Habrobracon eggs were x-rayed in first meiotic prophase (diplotene) and in late metaphase and allowed to develop parthenogenetically. Those treated in prophase have 50 per cent hatchability at about 12,000 r (lethal dose about 45,000 r); give an exponential hatchability curve which tends to become linear when dose is fractionated; may show, after treatment, fragments or bridges or both in division I, in division II or in both. Those treated in late metaphase have 50 per cent hatchability at about 400 r (lethal dose about 2,000 r); give a linear hatchability curve which does not change with fractionation of dose; may show fragments but no bridges in division I, either or both in division II. All eggs treated in either stage with lethal dose develop at least to first cleavage (20 per cent continue to blastoderm); show bridges and sometimes fragments in cleavage. A correlation of chromosome form, movement, and tension at time of treatment with sensitivity and cytological effects exists which suggests that x-ray injury is due to direct "hits" on chromosomes, and that sensitivity is associated with degree of tension to which chromosomes are exposed during irradiation; that nature of chromosome changes is due to their form and proximity during treatment. Lethal dose is not lethal to the treated cell (oocyte) but to its descendants (embryo) since chromosome fragmentation is not lethal, loss of fragments is.

*The problem of reversal of male haploidy by selection.* P. W. Whiting.

Except for the almost sterile, highly inviable diploid males of the wasp Habrobracon obtained in experimental cultures, diploid males are unknown in the Hymenoptera, as also in rotifers, thrips, mites except Mesostigmata, aleurodids and iceryine coccids, and in the beetle Micromalthus. It is probable that all normal males in these groups are haploid and that male haploidy has been attained in an evolutionary sense not more than six or seven known times. One of these attainments, taking place in an ancestral hymenopteron probably in the early Jurassic, has come to involve the entire order. Three conditions characterize male haploidy: (1) Production of males from reduced unfertilized eggs. (2) Reduction or omission of meiosis in spermatogenesis. (3) Complementary sex determination with heterozygous "double dominant" females. The problem of reversal of male haploidy is not to attempt to re-integrate any Jurassic protohymenopteran species, but rather to obtain by methods of genetics a strain of *Habrobracon juglandis* with normal biparentalism of males as well as of females. Inbreeding gives diploid males homozygous for sex. Selection has increased their viability from one to sixty per cent as compared with females. Cell size of diploid males is abnormally large, but is reduced somewhat in strains of high viability. Spermatogenesis of diploid males is of the haploid type, lacking chromosome synapsis and resulting in diploid sperm. If a strain with chromosome synapsis can be derived, it is considered that the problem can be solved, since sex determination should then shift from the complementary to the back-cross type with digametic females.

*Endomitosis in plants.* E. R. Witkus.

The process called endomitosis was discovered by Geitler in 1939 in insect material. During this process there is a chromosomal reduplication without a nuclear division, no spindle is present and there is no true anaphase movement of chromosomes. Throughout the whole process the nuclear membrane remains intact. Geitler divided the process into four stages, which he termed endoprophase, endometaphase, endoanaphase, and endotelophase. During endoprophase the chromosomes become shorter and thicker. The stage at which the chromosomes have reached their highest degree of contraction is called endometaphase. The nuclear membrane is intact and the chromosomes are not aligned on an equatorial plate. The SA-region of the chromosomes divides and the chromatids or now endoanaphase chromosomes slightly separate. After this separation the chromosomes undergo reversion to the resting stage. This reversion process occurs during endotelophase. The resulting cell then is tetraploid.

This process was also found to occur in the tapetal cells of *Spinacia oleracea* (Spinach) and apparently this is the first time that endomitosis, as defined by Geitler, has been reported for plant material.

The tapetal cells of *Spinacia* undergo two successive divisions during the early prophase stages of meiosis. The first division is an incomplete mitotic division resulting in binucleate

cells or in cells having dumb-bell shaped nuclei. The second of these divisions is in all cases endomitotic.

It becomes increasingly apparent that polyploidy brought about by a chromosomal reduplication without a nuclear division is of quite common occurrence in both plant and animal material. Endomitosis is only one of three known methods by which this can occur, although it has often been confused with all three in recent cytological literature. The first method is by a repeated reduplication in the resting nucleus as illustrated in the multiple complex cells of mosquito. The second is simply by a double reduplication in the resting nucleus as shown by certain cells in the root tips of polysomatic plants such as *Spinacia*. The third is by endomitosis.

It is interesting also to note that polyploidy arises by different methods in the root tip and tapetal cells of *Spinacia oleracea*.

### *A tetrahedral framework for native proteins?* Dorothy Wrinch.

It was suggested in 1936 that a fabric or atomic lamina is an essential element in the structure of native proteins and the lactim cyclol fabric was formulated as a working hypothesis (*Nature*, 137: 411). Today four different types of fabric—all necessarily polypeptide fabrics—are open for discussion; the lactim and enol cyclol fabrics, the hydrogen-bridged linear polypeptide fabrics, and the fabrics in which cyclized polypeptides are interlocked by hydrogen bridges (Jordan Lloyd and Wrinch, 1936; *Nature*, 138: 758; Astbury and Wrinch, *ibid.*, 139: 798; Wrinch, 1940; *Phil. Mag.*, 30: 64; 1941, 31: 177). This idea of an atomic lamina or fabric as characteristic of native proteins has been widely accepted and adopted. It has now been used to interpret the x-ray intensities in a study of horse methemoglobin in crystalline form.

We wish now to suggest that these (hol) intensities (Perutz, 1942; *Nature*, 150: 324) suggest not only laminae parallel to the c-planes (Boyes-Watson and Perutz, 1943; *Nature*, 151: 714) but also a second set of laminae at approximately the tetrahedral angle to the first. For this and a number of other reasons, the hypothesis is put forward that the native protein unit (of which there may be one, two, or more in the native protein particle) is built on a tetrahedral framework, the possibility that the enveloping truncated tetrahedron of the framework may be an octahedron not being excluded. In the case of horse methemoglobin, this suggestion implies trigonality of the individual frameworks about an axis approximately normal to the c-planes; thus it offers an interpretation of the fact that the lattice points and c-face centers in these planes form a triangular network which is very nearly equilateral. The hypothesis appears to bear very closely upon the fact that twins, trillings, and other compound crystals are extremely common in many different hemoglobins (Reichert and Brown, 1909; *The Crystallography of the Hemoglobins*, Washington, D. C.). This we propose for discussion the possibility that such situations as the apposition of tetrahedral frameworks by displacements plus rotations of (1)  $\frac{1}{6}$ ,  $\frac{2}{3}$  about the (111) axis; or (2)  $\frac{1}{6}$ ,  $\frac{1}{2}$ ,  $\frac{5}{6}$  about the (111) axis; or (3)  $\frac{1}{2}$  about the (110) or (112) axes, or  $\frac{1}{4}$ ,  $\frac{1}{2}$ ,  $\frac{3}{4}$  about the (100) axes, etc., are here realized. Attention is also directed to the obvious manner in which this postulate lends itself to the interpretation of the space groups and classes of crystal symmetries found in x-ray (Fankuchen, 1941; *Ann. N. Y. Acad. Sci.*, 41: 157) or classical studies of the native proteins.

# THE BIOLOGICAL BULLETIN

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## NATURAL HETEROAGGLUTININS IN THE SERUM OF THE SPINY LOBSTER, *PANULIRUS INTERRUPTUS*. II. CHEMICAL AND ANTIGENIC RELATION TO BLOOD PROTEINS<sup>1</sup>

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In a previous report (Tyler and Metz, 1945) it has been shown that lobster-serum contains at least ten heteroagglutinins for sperm or blood cells of various animals. Each of the heteroagglutinins was found to act on all the species tested that belong to the same group of animals. Since the group, in most instances, represents a taxonomic class, the heteroagglutinins are termed class-specific. The heteroagglutinins were found to be most probably protein, and by means of electrophoresis they were shown to be distinct from the hemocyanin which Allison and Cole (1940) and Clark and Burnet (1942) had considered to be the sole protein present in lobster-serum.

The relatively small amount found to be present accounts for Allison and Cole's conclusion which was based on approximate identity of the copper to protein nitrogen ratios of purified hemocyanin and of whole serum. Clark and Burnet's evidence was actually to the effect that there is no protein present with active antigenic properties different from that of pure hemocyanin. This is in accord with the results obtained with antisera prepared against heteroagglutinin by injecting rabbits with agglutinin that had been absorbed on rabbit cells. In the present paper a precipitation method for preparing the heteroagglutinins free of hemocyanin is described, and results of an electrophoretic examination of the material are presented. The agglutinating action of fibrinogen preparations from plasma and further serological tests are also reported.

### MATERIAL AND METHODS

Blood is quite easily obtainable from lobsters by means of a syringe inserted, between cephalothorax and abdomen, into the pericardial chamber. A twelve-inch lobster yields, in this manner, about 20 to 30 ml. of blood. For serum the blood was generally defibrinated by shaking with glass beads, filtered, and centrifuged; or it was occasionally allowed to clot, forced through a fine mesh wire screen, and centrifuged. For plasma the blood was drawn into a small amount of sodium

<sup>1</sup> This work has been aided by grants from the American Philosophical Society and the Rockefeller Foundation.

citrate solution, then subsequently filtered, centrifuged, and dialyzed against saline. One volume of 10 per cent citrate suffices to prevent fibrin-clotting in about 30 volumes of blood.

The agglutinative tests were made as previously described (Tyler and Metz, 1945) by mixing equal volumes of the sperm or blood cells (of sea-urchin, sheep, or other animal) and of serial two-fold dilutions of the test-solution adjusted to the appropriate salinity. Deviations from these proportions are specified in the tests.

#### EXPERIMENTAL PART

##### *Separation of heteroagglutinins from hemocyanin by isoelectric precipitation*

Hemocyanin was prepared from serum by isoelectric precipitation essentially as described by Allison and Cole (1940) and by Rawlinson (1940). This consists in dialysis against distilled water and then against dilute acetate buffer at the pH of the isoelectric point. Further purification is obtained by repeated solution in dilute ammonia and reprecipitation, by addition of acetate buffer (0.1 M., pH 4.5).

Rawlinson (1940), in the course of purification of hemocyanin from the *plasma* of the Australian spiny lobster, noted the presence of small amounts of protein which he considered to be fibrinogen. Such a non-hemocyanin protein is obtainable from the *serum* of the California spiny lobster, *Panulirus interruptus*.

When samples of serum or plasma of *Panulirus* were dialyzed against dilute, pH 4.5, acetate buffer, there invariably appeared small amounts of a pale precipitate that separated before the hemocyanin started to come down. The precipitates ranged in color from white to pink. After centrifugation, washing with distilled water and solution in dilute ammonia, the material was reprecipitated by slow addition of 0.01 M., pH 4.5 acetate buffer. The material was regularly found to start to precipitate at pH 5.0 and reach a maximum at pH 4.8. From the supernatants of the first precipitates the blue-colored hemocyanin was precipitated by continuation of the dialysis against the pH 4.5 buffer. The hemocyanin was obtained in crystalline form from concentrated solutions of it in dilute ammonia by the slow addition of dilute acetate buffer. Its precipitation was found to begin at pH 4.6 and to be complete at 4.5 to 4.4.

Samples of the purified hemocyanin and of the pale precipitate were tested for their ability to agglutinate the sperm or blood cells of various animals. After adjustment of the solution to appropriate pH and salinity by dialysis, they were tested on one per cent suspensions of the sperm of the polychaet, *Chaetopterus variopedatus*; the sea cucumber, *Stichopus californicus*; the starfish, *Pisaster ochraceus*; the sea-urchin, *Strongylocentrotus purpuratus*; the sea-squirt, *Ciona intestinalis*; and the grunion (smelt), *Leuristhes tenuis*; and of the erythrocytes of the sand bass, *Paralabrax maculatofasciatus*; the frog, *Rana pipiens*; the chuckwalla, *Sauromalus ater*; the pigeon, and sheep. The hemocyanin preparations, containing this material in amounts as great as or greater than normally present in the serum, were found to be completely inactive. The preparations of the pale, pH 4.8-5.0, precipitate gave very good agglutination of the cells of all the above named species.

Titer determinations were made with one of these preparations on sperm of *Strongylocentrotus*. In this case 0.2 ml. of serial two-fold dilutions of the solution were mixed with one drop of 10 per cent sperm-suspension. The protein con-

centration (from Kjeldahl nitrogen determination) of the solution was 0.7 per cent and its titer (minimum dilution giving definite microscopic agglutination) was 128. A sample of serum containing 5 per cent protein gave at the same time a titer of 256. This preparation showed, then, about  $3\frac{1}{2}$  times the activity of the whole serum.

*Electrophoretic examination of the pale precipitate<sup>2</sup>*

Another sample of the material freed of hemocyanin was reprecipitated at pH 5, dissolved in dilute ammonia, and dialyzed for 2 days in the cold against barbiturate buffer ( $\mu = 0.05$ ) at pH 7.7. It was then examined electrophoretically in the

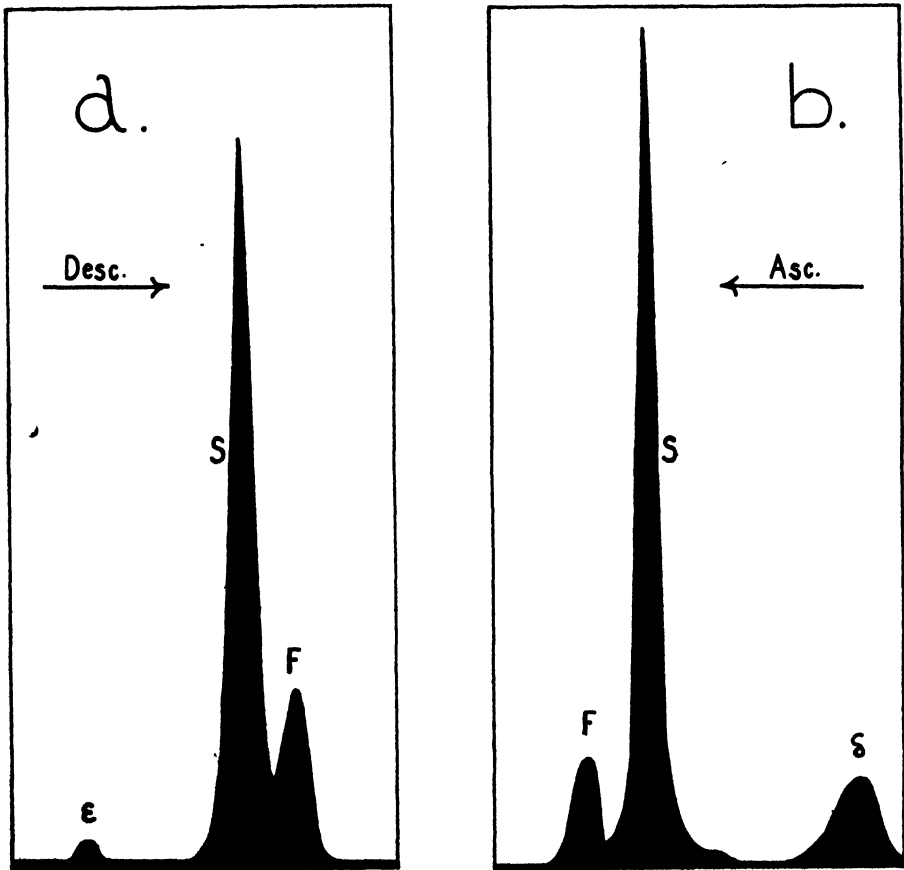


FIGURE 1. Electrophoretic patterns of pale (pH 5) precipitate from lobster-serum. *a*, descending (desc.) side; *b*, ascending (asc.) side; after 59 minutes of electrophoresis at pH 7.7, ionic strength 0.05 and 14.8 ma. Arrows show direction of migration. See text for further description.

<sup>2</sup> The apparatus employed was that constructed in the Division of Chemistry by Dr. Stanley M. Swingle to whom we are indebted for the electrophoresis of this material.

Tiselius' (1937) apparatus using the scanning method of Longsworth (1939). After 59 minutes of electrophoresis with a current of 14.8 ma., the patterns shown in Figure 1 were obtained. As may be seen from the figure two components, besides the  $\delta$ - or  $\epsilon$ -boundaries, are present in the serum. From the relative areas covered by the peaks the ratio of amount of slow component to that of fast component is approximately 5:1. At the end of the run the fast moving component was removed from the ascending side and the slow component (plus  $\delta$ ), from the descending side of the electrophoresis cell. After dialysis against normal saline, determinations were made of their agglutinative titers for rabbit cells and of the Kjeldahl nitrogen content. Samples of the original solution of the pale precipitate (taken from the cell after the run) and of normal lobster-serum were tested at the same time. The results are given in Table I. The nitrogen content of the solutions does not represent the relative concentrations of the components present in the original solution since there was some dilution with buffer upon their removal from the electrophoresis cell. As may be seen in Table I, the solution of the fast component showed no agglutinative activity for rabbit-erythrocytes although its nitrogen content was about one-third that of the slow component. The slow component proved highly active, giving almost twice the titer (per mg. N. content of solution) of the original solution and 24 times that of whole serum. This is approximately the order of magnitude of activity obtained (Tyler and Metz, 1945) for the components isolated by electrophoresis from whole serum.

TABLE I  
*Agglutinative titers of components obtained by electrophoresis  
of the pale (pH 5) precipitate from lobster-serum*

| Material                              | mg. Kjeldahl N.<br>per ml. | Agglutinative titer on<br>1% rabbit cells | Titer/mg. N. |
|---------------------------------------|----------------------------|---|--------------|
| Fast component (F of Figure 1)        | 0.29                       | 0   | 0            |
| Slow component (S of Figure 1)        | 1.008                      | 128                                       | 128          |
| Original solution (from cell-residue) | 3.85                       | 256                                       | 66.5         |
| Whole serum                           | 11.97                      | 64  | 5.3          |

The slow component obtained here was also tested on cells of all the animals listed on page 194, with the exception of *Sauromalus* and *Leuristhes*. It proved to be highly active with all of them. In the previous report lobster-serum was shown to contain at least ten "class-specific" heteroagglutinins. It is evident from the present results that these are represented by a single electrophoretic component of the serum, unless there is some active component in the stationary  $\delta$ - or  $\epsilon$ -boundary. The latter is, however, highly unlikely since the original material for the present test was obtained by precipitation at pH 4.8 to 5.0 and the electrophoresis was run at pH 7.7. For any material to remain in these stationary boundaries it would have to be isoelectric at the latter pH.

#### *Preparation of fibrinogen and tests for heteroagglutinating activity*

Lobster-plasma upon being brought to 25 per cent saturation with ammonium sulfate formed a white to pink precipitate which separated easily upon centrifuga-

tion. The precipitate was washed with distilled water and dissolved in sea water. Addition of fresh lobster-blood-cells to the solution caused it to form a firm clot. A pH 5.0 precipitate obtained directly from plasma was found to contain fibrinogen, which could be separated from the remaining protein material by precipitation with ammonium sulfate. None of the preparations from serum were found to contain fibrinogen.

TABLE II  
*Agglutinative titers of protein preparations from plasma and serum*

| Material                   | mg. Kjeldahl N.<br>per ml. | Agglutinative titer on<br><i>Strongylocentrotus</i> sperm | Titer per mg. N. |
|----------------------------|----------------------------|---|------------------|
| Fibrinogen preparation (I) | 1.25                       | 32 to 64  | 26 to 51         |
| Pale precipitate (II)      | 1.25                       | 64 to 128   | 51 to 102        |
| Hemocyanin                 | 7.4                        | 0   | 0                |
| Whole serum                | 8.5                        | 128 to 256  | 15 to 30         |
| Plasma                     | 8.5                        | 256 to 512  | 30 to 60         |

A fibrinogen preparation (I) was obtained from whole plasma by 25 per cent saturation with ammonium sulfate. The precipitate was dissolved and reprecipitated by dialysis to pH 5.0. This preparation was tested for agglutinating action on sperm of *Strongylocentrotus* in the same manner as on page 194. The supernatant from the 25 per cent ammonium sulfate precipitate was dialyzed against tap water and then brought to approximately pH 5 by dialysis against pH 4.5 buffer. This gave a pale precipitate (II) which resembled the pale precipitate from serum. After solution and dialysis against sea water it, too, was tested for agglutinating activity. The results are given in Table II along with simultaneous tests of whole serum, plasma, and hemocyanin. The presence of calcium in the sperm suspension does not interfere with the tests, since clotting of the fibrinogen does not occur unless fresh lobster-blood-cells are added. As the table shows, plasma has about twice the agglutinating activity of serum. The fibrinogen preparation proved about half as active as the pale precipitate.

Another pale precipitate was also obtained directly from plasma by dialysis against pH 4.5 buffer. When the precipitate was dissolved and brought to 25 per cent saturation with ammonium sulfate there separated out some material that proved to be fibrinogen. It appears from the experiments reported above that the isoelectric point of fibrinogen is not greatly different from that of the heteroagglutinin found in serum. This conclusion was verified by Mr. Maurice Rapport, who repeated some of our experiments, and made an electrophoretic examination of plasma and of protein preparations separated from plasma. The pH 5.0 precipitate from plasma showed two electrophoretic components, the patterns being similar to those of Figure 1. The smaller, faster component probably corresponded to the fast component observed in serum preparations. The other component, containing agglutinating activity, could not be separated further during 100 minutes of electrophoresis at pH 7.3, 1.2° C. and 20 ma.

Precipitation of the pH 5.0 precipitate from plasma with ammonium sulfate at 40 per cent of saturation removed nearly all of the agglutinating activity, but left behind a small amount of protein material. The ammonium sulfate precipitate

contained 3.5 mg. Kjeldahl N./ml, and had a titer of 64 against *Strongylocentrotus* sperm. The supernatant contained 1.6 mg. N./ml, and titrated only to 4. Mr. Rapport showed that this small residue migrated rapidly in the electrophoresis apparatus at pH 7.3. It probably corresponded to the fast component from serum.

In the absence of more exhaustive chemical and electrophoretic separations it is not possible to decide with certainty whether the agglutinative activity found in fibrinogen preparations is associated with fibrinogen itself, or is due to the presence in these preparations of the heteroagglutinin fraction which is present in serum.

### *Antigenic relationship of the blood proteins*

Two rabbits that were each given two courses of intravenous and intra-abdominal injections with a total of 375 mg. of purified hemocyanin produced very good precipitating antisera. The titers (end point of precipitation on mixing equal volumes of antiserum and serial dilutions of a 10 per cent hemocyanin solution) ranged from 10,000 to 20,000 in terms of antigen dilution and optimal proportions (second optimum, see below) were obtained at approximately one volume of 10 per cent hemocyanin to 10 to 20 volumes of antiserum. The antisera also reacted very well with whole lobster-serum, the optimal proportions point being about 9 volumes of antiserum to one volume of the lobster-serum.

Tests were then made of the ability of antiserum vs. hemocyanin to remove natural heteroagglutinin from whole lobster-serum. One volume of lobster-serum was absorbed with 9 volumes of the rabbit antiserum and the supernatant tested for ability to agglutinate rabbit-erythrocytes and *Strongylocentrotus* sperm. The absorbed serum gave no reaction with these cells, while control lobster-serum gave good agglutination out to dilutions of 1/90 (+ + + reaction) with the rabbit cells and 1/80 (+ reaction) with the *Strongylocentrotus* cells respectively.

It appears, then, that antibodies prepared against hemocyanin also react with the natural heteroagglutinins present in lobster-serum.

One of the anti-hemocyanin rabbit sera was also titrated with the solution of electrophoretically purified heteroagglutinin (slow component). A titer (dilution of antigen) of 128 was obtained for this solution which contained one mg. Kjeldahl N. per ml. A control hemocyanin solution containing 8 mg. N. gave a minimum titer (end point not reached) of 4096, or 512 per mg. N.

Another antiserum against hemocyanin was also titrated with various protein fractions separated from lobster-blood. The titer (dilution of antigen) of reprecipitated hemocyanin was 20,000 for a solution containing 6.6 mg. Kjeldahl N. per ml. or 3000 per mg. N. For the heteroagglutinin (pH 5 precipitate from serum, reprecipitated), the titer was 200 for a solution containing 1.6 mg. N. per ml. or 125 per mg. N. For the fibrinogen (ammonium sulfate precipitate from plasma), the titer was 200 for 3.4 mg. N. per ml. or 60 per mg. N.

In these titrations, it was sometimes noted that precipitation occurred in the first few tubes, containing concentrated antigen solutions. In intermediate dilutions, no precipitation occurred, but a second zone of precipitation appeared in the higher dilutions. This was noted both with hemocyanin and fibrinogen, but not with the agglutinin preparation (pale precipitate from serum) used. Boyden and deFalco (1943) reported a similar double zone phenomenon with *Homarus* serum titrated against anti-*Homarus*-hemocyanin. They pointed out that this is indicative of the

presence of two kinds of antibodies in the antisera. However, this does not seem to be the entire explanation, since we find that absorption of a sample of antiserum with an amount of hemocyanin which corresponds to the lower of the two optima removes all antibody for the homologous antigen, as well as for fibrinogen and pale precipitate.

Two rabbits were also immunized with whole lobster-serum, each receiving a total of 5.5 ml. of serum in two courses of three injections each, with three weeks rest between courses. The antisera obtained one week after the last injection gave very good precipitation with the homologous antigen, optimal proportions (second optimum) being obtained with one volume of lobster-serum to approximately 16 volumes of antiserum. A sample was absorbed with purified hemocyanin and tested on whole serum, a concentrated solution of the pale (pH 5) precipitate, and a fibrinogen preparation. It failed to give precipitation with any of these antigens. This confirms the findings of Clark and Burnet (1942) and indicates that the other blood proteins have no active antigenic groups other than those present in the hemocyanin. Alternatively, the results might be explained on the basis of competition of antigens (see Sachs, 1929), such that the rabbit does not form antibodies against other antigens when one powerful antigen (the hemocyanin) is present in excess in the material (whole lobster-serum) used for immunization. However, in view of the analogous results obtained (Tyler and Metz, 1945) with antisera prepared against heteroagglutinin, and with antihemocyanin sera (above), the alternate explanation seems highly unlikely.

#### SUMMARY

1. Lobster-serum contains small amounts of other protein constituents besides hemocyanin.
2. The "class-specific" heteroagglutinins of lobster-serum are found to reside in a component that is obtained free of hemocyanin by isoelectric precipitation at pH 4.8 to 5.0.
3. Electrophoresis of this "pale precipitate" reveals the presence of two components, of which the more slowly migrating one bears the heteroagglutinating activity. The ten separate "class-specific" heteroagglutinins are thus evidently represented by a single electrophoretic component.
4. There is some indication that fibrinogen obtained from the lobster plasma may also act as heteroagglutinin.
5. Antibodies produced in rabbits against purified hemocyanin also react with the slow electrophoretic component (heteroagglutinin) of the pale precipitate and with fibrinogen. Absorption tests with antisera vs. whole lobster-serum fail to reveal the presence of any specific antigenic groups other than those of the hemocyanin. The other blood proteins are, then, evidently serologically equivalent to hemocyanin.

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# STUDIES ON MARINE BRYOZOA. I. AEVERRILLIA SETIGERA (HINCKS) 1887

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## INTRODUCTION

During the summer of 1944 collections of *Aeverrillia setigera* were made at New Bedford and Woods Hole, Massachusetts. Perusal of literature pertaining to this species showed that a more complete account of this form would not be amiss. This article brings together all available distribution and anatomical data previously given for this form and adds to it some new distribution data, more complete illustrations than were heretofore available and a considerable amount of anatomical and some ecological data.

The writer wishes to acknowledge, with sincere appreciation, the kindness of Dr. Hannah Croasdale of Dartmouth College and of the Marine Biological Laboratory of Woods Hole, Mass., who collected the first specimens of *A. setigera* from New Bedford, Mass., and turned them over to the writer for study, and to Dr. Raymond C. Osburn of the University of Southern California who so kindly checked the specimens, confirming the identification and who offered many helpful suggestions.

## DISTRIBUTION

The species *Buskia setigera* has been reported previously by the following authorities from the localities listed below:

Hincks, 1887 (pp. 121, 127-128; Pl. XII, Figs. 9-13), from the Gulf of Bengal, around the Mergui Archipelago.

Kirkpatrick, 1890a (pp. 603, 612), between Australia and New Guinea in the Torres Straits, 20 miles off Warrior Island.

Kirkpatrick, 1890b (p. 17), off Tizard Banks in the China Sea.

Thornely, 1905 (p. 128), from Ceylon.

Harmer, 1915 (pp. 87-88; Pl. 5, Figs. 8-10), from the Bay of Bima (India), Bay of Badjo, west coast of Flores (Malay Archipelago), Makassar, Borneo Bank, off Pulu Jedan, east coast of Aru Islands, and also in the following unnamed locations: Station 164, at  $1^{\circ}42'.5$  S.;  $130^{\circ}47'.5$  E.; Station 166, at  $2^{\circ}28'.5$  S.;  $131^{\circ}3'.3$  E.

Thornely, 1916, off Poshetra Head, Kattiawar, and Ceylon.

Hastings, 1927 (p. 351), at Menzaleh Lock and other stations at the Suez Canal.

Livingstone, 1927 (p. 67), from Queensland, Australia.

Hastings, 1932 (p. 407), from Penguin Channel and N. E. Low Island, Great Barrier Reef, Australia.

Osburn, 1933 (p. 64), from Porto Rico.

Marcus, 1937 (p. 143; Pl. 29, Fig. 76), from Bay of Santos, Brazil, South America.

Osburn, 1940 (p. 343), from Porto Rico.

Hutchins, 1945 (p. 539), Pine Orchard, Long Island Sound, Connecticut, U.S.A.

Additional discussion of the species occurs in the following articles:

Osburn and Veth, 1922; (p. 159).

Marcus, 1938; (p. 61).

Marcus, 1939; (pp. 168, 171).

Marcus, 1941; (pp. 74-77, 147; Pl. X, Fig. 45).

The above reports indicate that the species is distributed near several continents, —Africa (Suez Canal), Asia, Australia, South America, and North America, and also around several islands, including Porto Rico. The present article reports its occurrence around the State of Massachusetts, extending the northerly range of this species to  $41^{\circ}38'$  N. Latitude.

*Averrillia setigera* was found in two Massachusetts localities. The first collection was made by Dr. Hannah Croasdale on July 29, 1944, at Black Rock in the Harbor of New Bedford, Mass. The next collections were made by the author on August 4, 13, and 14, 1944, at Stony Beach, Woods Hole, Mass. Further details of the nature of the collecting site and the associated biota will be given in the ECOLOGY section.

#### ECOLOGY

The New Bedford Harbor specimens were collected by Dr. Croasdale at the time of low tide, from the littorine region around Black Rock, along with red algae, at a depth of less than 2 feet below the surface of the water. The Woods Hole specimens came from a large, partially submerged boulder located approximately 50 yards from shore. The sea bottom around the boulder is largely sand although there are some rocks a short distance away on each side of the boulder. The general locality is not subjected to strong wave action. The boulder is almost completely submerged at high tide but is about half exposed at low tide. Its sides are well covered with algae of various kinds as well as with a luxuriant fauna. The *A. setigera* colonies were collected at low tide, a foot or two below water level, by gathering likely looking *Chondrus* and *Ascophyllum* algae off the boulder.

The Woods Hole *A. setigera* specimens were found growing in close association with the following animal forms: *Folliculina*, *Vorticella*, *Sycon*, *Obelia*, *Sertularia*, other hydroids, *Bowerbankia gracilis*, *Bugula flabellata*, *Crisia eburnea*,

*Hippothoa hyalina*, *Pedicellina cernua*, and *Stephanosella biaperta*. The *Aeverrillia* autozooids, and in some instances stolons, had a few *Folliculina*, *Vorticella*, or *Pedicellina*, growing on them. The *A. setigera* colonies grew on hydroid stems and on the same algal thalli (*Chondrus* and *Ascophyllum*) as *Bugula flabellata*, *Hippothoa hyalina*, *Crisia*, and the other Bryozoa.

*Aeverrillia setigera* has been collected from varying depths, from one or two feet below tide mark (present author) to much greater depths (other writers). Kirkpatrick found specimens at depths of  $5\frac{1}{2}$  and 27 fathoms; Thornely (1916), at 7 fathoms; Hastings (1932), at 8 to  $15\frac{1}{2}$  fathoms; Marcus (1937), at 17 meters; while Harmer found specimens at greater depths: 0 to 40 meters, 55 meters, 59 meters and 118 meters.

This Bryozoan grows on the following types of substratum: 1, on broken shells (Kirkpatrick, 1890a); 2, on stems of *Idia pristis* (Thornely, 1916); 3, on stems of hydroids and Bryozoa (Osburn, 1940); 4, on stems of *Nellia oculata* Busk (Hincks, 1887); 5, on hydroids and the following Bryozoa: *Bugula*, *Catenicella* and *Valkeria atlantica*, which were dredged from areas whose bottom consisted of such materials as mud, sand, hard coarse sand, coral, shells, and stones (Harmer, 1915); and 6, on hydroids like *Obelia* and algae like *Chondrus* and *Ascophyllum*, in close association with many other already mentioned animal forms (present paper).

#### DESCRIPTION OF SPECIES

The status of Bryozoa as an entire group is still an unsettled problem. It has been considered a Phylum, a Sub-phylum and a Class. Each category has its earnest and qualified supporters. With this in mind the following taxonomy of the *Aeverrillia* species, patterned after the work of Dr. Marcus, is given:

————— BRYOZOA Ehrenberg 1831  
 Class ECTOPROCTA Nitsche 1869  
 Order GYMNOLAEMATA Allman 1856  
 Sub-order CTENOSTOMATA Busk 1852  
 Group STOLONIFERA Ehlers 1876  
 Family Valkeriidae or Mimosellidae?  
 Genus *Aeverrillia* Marcus 1941  
 Species *setigera* Hincks 1887

The classification of *Aeverrillia setigera* has undergone a few changes since its original description by Hincks in 1887. Its generic names were *Buskia*, *Hippuraria*, and now *Aeverrillia*. The latter genus was erected in 1941 by Dr. Marcus in honor of A. E. Verrill.

The question regarding the family into which it should be placed is set forth by Marcus (1941, p. 147) thus: "*Aeverrillia* does not need a new family; the genus can be placed in the Valkeriidae or perhaps in the Mimosellidae as now enlarged by Bassler (1935, p. 8)." Earlier the species had been placed among the Triticellidae, the Buskiidae, and eventually into the Valkeriidae.

The colonies are delicate yellowish or very pale amber colored transparent traceries closely adherent to various living and non-living submerged objects. They are barely big enough to be seen with the unaided eye. They consist of paired

individuals connected by slender stolons. The stolons and individuals are chitinized and firm-walled. The stolons especially have a well thickened wall.

Bryozoa exhibit polymorphism. The *Aeverrilla* colony consists of three types of structures or possibly individuals, namely stolons, peduncles, and autozooids.

In the colony there is a main or primary axis or stolon and lesser (secondary and sometimes tertiary) stolons (Fig. 7). The lesser stolons are more apparent in older colonies than in young ones.

These stolons, according to Dr. Marcus, are composed of kenozoöecia. The long slender tubular kenozoöecia of each stolon grow longitudinally and are attached end to end. Those of the secondary stolons have their origin at the sides of the primary stolon usually with a peduncle intervening between the primary and secondary stolons. The tertiaries have their origin at the sides of the secondaries, likewise usually with an intervening peduncle. Some stolons appear to arise directly from other stolons without an intervening peduncle (Fig. 8). Also, one of a pair of opposite stolons may arise from a stolon without the intervention of a peduncle while its partner may have a peduncle between it and its parent stolon (Fig. 8). Whether this barrenness of one stolon may be a temporary or a permanent condition is uncertain.

The primary and secondary stolons are at right angles, approximately, to each other but it is difficult to say the same about the tertiaries because the latter are sometimes twisted, gnarled, and not often found running in a straight line because of the limited area of the substratum on which the specimens grow. The secondary stolons usually originate in pairs, one stolon on each side of the primary stolon and directly opposite the other, growing away from each other.

The primary stolons, possibly because they are older, have thicker walls than the secondary stolons. The primaries are also somewhat straighter than the secondary and tertiary stolons but that again may be due to the limited substratum. Anastomoses occur occasionally, especially where there are tertiary and secondary stolons over a crowded or limited substratum. Hincks suggested the possibility of anastomosis of branches.

Primary stolons are very closely and entirely adherent to the substratum which in many cases proves to be a hydroid stem or *Chondrus* or *Ascophyllum* thallus. The primaries follow the stems or thalli in a fairly straight line for some distance. The secondaries and tertiaries must find what surface they can. Some of the lesser stolons look as if they are not necessarily attached along their entire length.

Generally the kenozoöecia of the stolons are slightly enlarged distally at the point of origin of the lateral kenozoöecia or peduncles. Transverse uniporous septa mark the proximal and distal limits of the kenozoöecia along the stolons (Figs. 2 and 10). There are septa also at the points of origin of the lateral branches on the main stolons (Figs. 16 and 18). The region of the septum is sometimes referred to as the node and the stretch of stolon between two transverse septa, as the internode.

Stolon length is variable (Figs. 7 and 9, Table I). Some secondary stolons are short, some long. Some tertiary stolons are considerably longer than the primaries or than some of the secondaries. Stolon diameter is given in Table I.

The stolons under low power observation ( $75\times$  magnification) appear empty or tubular but under higher magnification ( $430\times$ ) a cellular lining membrane is evident within them.

TABLE I

*Measurements of Massachusetts specimens of Aeverrillia setigera*

| Part  | Number of readings | Maximum   | Minimum   | Average   | Refer to Figs. |
|---|--------------------|-----------|-----------|-----------|----------------|
| A. Length or height of furled setigerous collar                                 | 17                 | 0.602 mm. | 0.440 mm. | 0.531 mm. | 6, 17          |
| B. Diameter at distal end of unfurled setigerous collar                         | 5                  | 0.537 mm. | 0.370 mm. | 0.440 mm. | 6              |
| C. Diameter at the basal, proximal end of the setigerous collar                 | 6                  | 0.110 mm. | 0.059 mm. | 0.083 mm. | 6, 17          |
| D. Length of orificial spine  | 20                 | 0.259 mm. | 0.141 mm. | 0.204 mm. | 6              |
| E. Diameter of extruded vestibular membrane                                     | 1                  |           |           | 0.111 mm. | 6              |
| F. Length of extruded vestibular membrane                                       | 1                  |           |           | 0.321 mm. | 6              |
| G. Length of tentacular sheath  | 1                  |           |           | 0.237 mm. | 6              |
| H. Diameter of tentacular sheath  | 2                  | 0.096 mm. | 0.074 mm. | 0.085 mm. | 6, 11          |
| I. Autozoid width at widest part  | 7                  | 0.212 mm. | 0.170 mm. | 0.185 mm. | 8              |
| J. Autozoid length from base of zoid to base of orificial spines                | 18                 | 0.592 mm. | 0.481 mm. | 0.552 mm. | 8              |
| K. Stolon diameter, at the normal thickness, not the swollen area of the stolon | 24                 | 0.049 mm. | 0.015 mm. | 0.027 mm. | 8              |
| La. Length of shorter lateral surface of the clasping processes                 | 23                 | 0.179 mm. | 0.043 mm. | 0.110 mm. | 7              |
| Lb. Length of longer lateral surface of the clasping processes                  | 23                 | 0.182 mm. | 0.077 mm. | 0.119 mm. | 7              |
| M. Width of stolon at most swollen part, near node                              | 18                 | 0.051 mm. | 0.034 mm. | 0.040 mm. | 8              |
| N. Length of peduncle   | 19                 | 0.170 mm. | 0.068 mm. | 0.114 mm. | 18             |
| O. Diameter of peduncle   | 19                 | 0.071 mm. | 0.039 mm. | 0.058 mm. | 8              |
| P. Length of internode  | 24                 | 1.013 mm. | 0.294 mm. | 0.658 mm. | 9              |
| S. Number of tentacles  | 6                  | 8         | 8         | 8         | 6, 13, 14      |
| T. Number of setae in setigerous collar   | 3                  | 19        | 16        | 17        | 6              |

## PLATE I

All figures except Figures 2 and 6 have been drawn with the aid of a camera lucida. All are of *Aevertillia setigera*.

FIGURE 1. A chitinized sponce of the proventriculus, seen from the lumen side. Note the converging rows of teeth. Drawn to the same scale as Figure 10. This and Figures 3, 4, and 5 are from gizzard remains found in empty, degenerated autozoecia.

FIGURE 2. Detail of the uniporous septum which occurs along the stolons.

FIGURE 3. Latero-basal view, from the concave side of the chitinized gizzard sponce. All the softer parts of the gizzard have disintegrated, leaving only the hardened plate or sponce. Drawn to the same scale as Figure 10.

FIGURE 4. Side view of a somewhat flattened chitinized gizzard sponce. Drawn to the same scale as Figure 10.

FIGURE 5. Side view of a chitinized gizzard sponce of the more usual shape. Some of the teeth are darker than others. Drawn to the same scale as Figure 10.

FIGURE 6. A diagram showing several things: the relation between the open or unfurled setigerous collar, the eight tentacles, three of the four zooecial spines around the orifice and the lettered areas A through G along which measurements for Table I have been made. The same letters are found listed in the first column of the Table.

Line A stands for the length or height of the setigerous collar. It was measured only when furled or very slightly unfurled.

Line B represents the diameter of an unfurled setigerous collar, at its distal, expanded end.

Line C represents the diameter at the basal or proximal end of the setigerous collar.

Line D represents the length of the orificial spine.

Line E represents the diameter of the vestibular membrane and the area it encloses.

Line F represents the length of the vestibular membrane.

Line G represents the length of the tentacular sheath or the distance between the lophophore and the base of the setigerous collar, C—C.

FIGURE 7. Part of an old zoarium or colony showing the growth habit, anastomosis of branches (AN), primary stolon (P.ST.) and secondary stolon (S.ST.). All the autozoecia are empty of polypides. One (Z) has the setigerous collar in place yet but has no polypide. Some of the zooecia have two or three acuminate processes (B.P.). Measurements of these acuminate processes were made along two surfaces, the shorter (La) and the longer (Lb). These figures are to be found in Table I. The "membranous" area mentioned by Hincks is not very plain on most specimens. However, there is a hint of it in the second and fourth zooecia from the top. Drawn from freshly collected material on Aug. 14, 1944, and to the scale shown at its base.

FIGURE 8. An autozoecium attached to a very long secondary stolon. The location of certain measurements mentioned in Table I is indicated on the drawing.

Line I represents the diameter of the autozoid.

Line J represents the length or height of the autozoid exclusive of spines.

Line K represents the diameter of the stolon along most of its length and not at the swollen areas.

Line M represents the diameter of the stolon at the slightly swollen node region.

Line O represents the height or thickness of the peduncle.

The faintly curving line along the autozoecium suggests the location of the "membranous" area. The scale below belongs with this sketch.

FIGURE 9. Part of a colony showing four autozoids growing quite regularly in pairs, on peduncles at opposite sides of the primary stolon. Two are empty, the third has a setigerous collar and the fourth has a living polypide within. A darker gizzard is evident within the last. The scale directly below belongs to this colony.

FIGURE 10. The section of the stolon showing a septum and the swollen part represented by M in Figure 8. This is the region of the node. Drawn to the scale directly below. Figures 1, 3, 4, 5, and 11 also are drawn to this scale.

FIGURE 11. Part of an autozoid showing the basal region of the setigerous collar through which is lightly indicated the tentacle-bearing lophophore and tentacular sheath. At the base of the setigerous collar are placed two small letters H which represent the width of the tentacular sheath. The measurements are found in Table I. Below the setigerous collar is the vestibular membrane through which are visible muscle fibers. Drawn to the same scale as Figure 10.

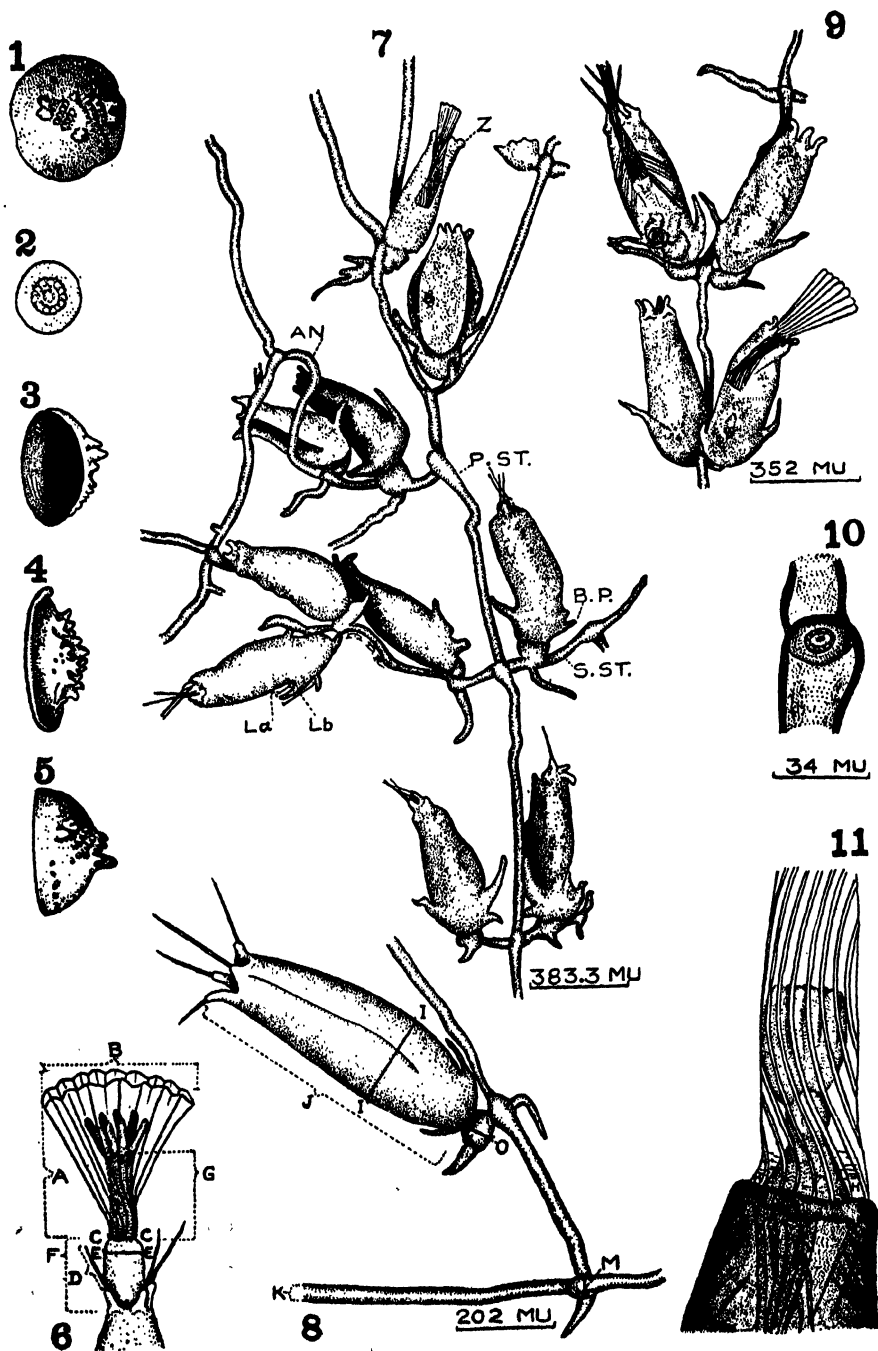


PLATE I

The second type of structure or possibly individual (?) in *A. setigera* is the peduncle, so designated by Marcus (1937, p. 142). This is a short much swollen segment generally placed between stolons which are at right angles to each other and found at the base of the autozooids (Figs. 8 and 16). It originates from a stolon and gives rise to a stolon and an autozoid. It is cut off from the stolons and autozoid by a uniporous septum. A peduncle is more swollen and of shorter length than the stolon kenozoecium and has a lining membrane. In one instance there appeared a few transverse fibers inside a peduncle.

The third type of individual in an *A. setigera* colony is the autozoid. It arises from the peduncle. The autozooids are just big enough to see with the unaided eye. Harmer (1915, p. 87) gave their length as 0.48–0.55 mm. and Osburn (1940, p. 343) as 0.50–0.60 mm. Their width was given as 0.18 mm. (Harmer, 1915 and Osburn, 1940). Measurements of the Massachusetts specimens are given in Table I.

The autozooids occur in pairs bilaterally placed with respect to the primary stolon (Fig. 9). Where secondary stolons are well developed the autozooids occur in the same manner along the secondary stolon. Occasionally one of the paired autozooids is missing but a stub of its peduncle or a stolon may be present in its place (Fig. 8). These paired autozooids are not truly parallel but converge slightly basally as shown

## PLATE II

All figures are drawn with the aid of a camera lucida and are of *Aevertillia setigera*.

FIGURE 12. A part of the unfurled setigerous collar, showing the delicate transparent membrane which folds like a fan. Its stiff supporting ribs or setae are transparent also. Drawn to the scale at left.

FIGURE 13. An autozoid in which a very young polypide is growing. A characteristic setigerous collar is not yet present although its Anlage (SC) is visible. Eight tentacles can be counted. The digestive tract is small. A gizzard or proventriculus is present in it. Drawn from living material on August 13, 1944, to the scale shown directly below.

FIGURE 14. Another young autozoid but slightly older than that of the preceding figure. Drawn to the same scale.

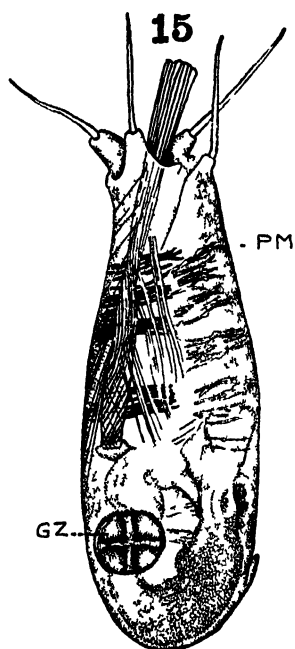
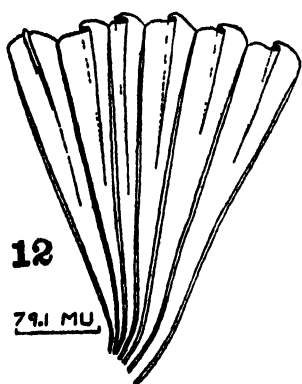
FIGURE 15. View of a mature autozoid showing an almost completely retracted polypide, a very long folded setigerous collar partially withdrawn, the U-shaped digestive tract twisted around in the lower half of the zoecium. The gizzard (GZ) is oriented in such a manner that one is looking along its vertical axis. Some of the body wall and polypide musculature is shown, particularly the circularly arranged parietal muscles (PM). The acuminate process is barely visible. Drawn to the same scale as Figure 12.

FIGURE 16. A partly retracted autozoid. The tentacle tips are just barely visible in the dark mass at the base of the spine-bearing processes. The somewhat indistinctly depicted digestive tract is in the basal part of the zoecium. Only a part of the autozoid at left is shown. The scale directly above the setigerous collar applies to this figure.

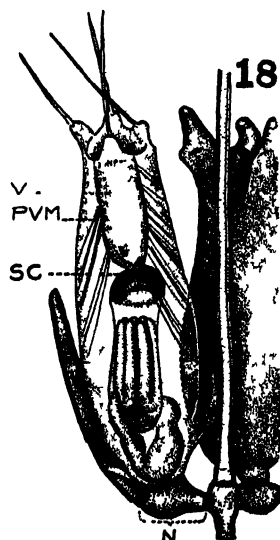
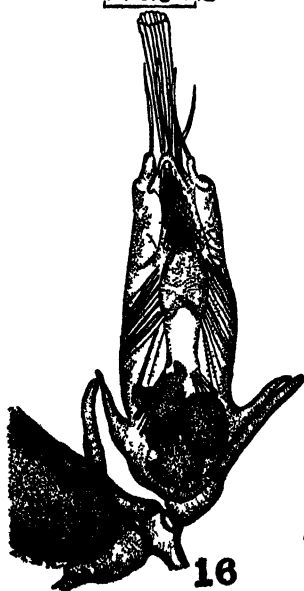
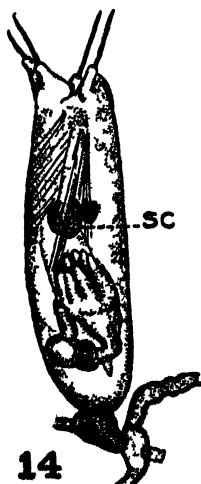
FIGURE 17. A folded setigerous collar showing typical twisting of the supporting ribs or setae. The transparent membrane is faintly indicated at the distal end. Drawn to the scale directly below.

FIGURE 18. A young autozoid is shown at left. Only a part of the right one is included. The young polypide has eight tentacles and a U-shaped digestive tract. The setigerous collar is not visible but its Anlage (SC) is present. The vestibule (V) and the parieto-vaginal muscles (PVM) are plain. Line N represents the length of the peduncle which bears the autozoid. Measurements of it are given in Table I. Drawn to the same scale as Figure 13, on Aug. 13, 1944, from fresh material.

FIGURE 19. Three of the four chitinized gizzard sponces. The teeth are darker than the rest of the disc in this particular case. Muscle fibers encircle the cluster of four sponces and are here indicated by horizontal or parallel lines. Drawn to the scale at left.



146.8 MU



34 MU



in Figure 9. They are not upright but are recumbent at an angle close to the substratum. The basal part rests directly on the substratum, or is very close to it, while the distal part is free. The autozooids are somewhat elongate ovate with the broad end attached. The side nearest the substratum and the inter-autozooidal stolon is slightly flatter than the opposite side. At its point of origin the autozoid may be globose as in Figure 14 or slightly "stemmed" as in the right-hand individual of Figure 18.

The lower half of the autozoid is swollen slightly. From it arise from one to four, usually two, acuminate clasping processes (Figs. 7 and 16), which were called "tubular adherent processes" by Hincks (1887, p. 128) and "spines" by Harmer (1915, p. 87). They are placed obliquely on the zoid. They may touch the stolons or the neighboring autozoid or else cling to the substratum without touching either the adjacent autozoid or the stolon. Colonies in place on hydroids show some of these clasping processes curling around the hydroid stems, closely adherent. These clasping processes are hollow and not separated by any sort of septum from the rest of the zoid.

Hincks (1887, p. 127) described a large aperture closed by a membranous wall on the greater part of the ventral side of the autozoid. It is difficult to see in the Massachusetts specimens although indications of it are present in Figures 7 and 8. In Figure 7, it is evident on the second and fourth autozooids from the top. Moreover, it appears chitinated rather than membranous.

The distal tapering end of the autozoid has four spine-bearing processes (basal segments or flaps). Occasionally more than four flaps may occur. Harmer (1915, p. 88) reported a specimen with eight. This condition however is very infrequent. These flaps are arranged around the zooecial orifice through which the setigerous collar may be protruded.

The position of these distal triangular flaps is not rigidly, immovably fixed. The line of bending is at the base of the triangle. Sometimes the flaps may be flexed inward so that their spines may cross each other above the orifice as in the top left-hand zoid of Figure 9, or in Figure 16. This is the usual position when the setigerous collar is withdrawn into the autozoid. When the setigerous collar is out the flaps are bent outward as in Figure 6. This is the condition also in many empty zooecia. Whether there are any muscle fibers controlling the movement of these flaps was impossible to determine from the material at hand.

The flaps are more heavily chitinated than the surrounding zooecial wall. The difference is quite noticeable.

The apex of a triangular flap is rounded in all views. A sharply tapering, slightly irregular orificial spine is set shallowly into this rounded area. The spine is hollow, but so far as it is possible to determine its cavity is not continuous with the cavity of the flap but is cut off by a septum. In Porto Rican specimens the spines measured 0.20–0.30 mm. (Osburn, 1940, p. 343). Measurements of Massachusetts specimens are given in Table I.

The setigerous collar is long and very slender when furled. Harmer (1915, p. 88) gives its length as 0.46 mm. and its breadth at the distal end as 0.130 mm. This last figure is undoubtedly of a partly furled individual. The dimensions of the Massachusetts specimens are included in Table I.

The setigerous collar can be protruded clear out of the autozoid (Fig. 6). On the other hand, it also can be completely withdrawn into the autozoid. In fact it

can be pulled in so far that its uppermost or distal tip is halfway down inside the zoid. There are muscular fibers attached to its base (Fig. 11). When it is completely withdrawn the tentacles are below it. When it is protruded and expanded the tentacles are within its circle of setae (Fig. 6).

Hincks (Pl. XII, Fig. 13), Harmer (Pl. V, Fig. 9), Marcus (1937, Pl. XXIX, Fig. 76) and the present writer (Figs. 6, 11, 15, and 17) have pictured the peculiar spiral twisting of the setae of the collar. The setae reinforce a delicate, colorless, transparent membrane which folds neatly like a fan along scarcely discernible creases between adjacent setae, when the collar is being withdrawn (Figs. 6, 12, and 17). The setae or ribs supporting the collar are extremely regular in diameter from base almost to the very tip.

The collar is often found in excellent condition even when all the zoid contents except the zoecial wall have disintegrated.

In young zoids as represented in Figures 13, 14, and 18 the setigerous collar is not yet completed but is represented by a mass of germinative tissue, SC, which temporarily forms a flexible canopy above the tentacles, at the bottom of the vestibule.

The vestibule is the cavity down which the setigerous collar travels when being withdrawn. Its wall is formed by a soft vestibular membrane, to which are attached a number of fibers which constitute the parieto-vaginal muscles. The vestibular membrane is shown withdrawn or introverted in Figure 18 and extruded in Figure 6.

The circular lophophore bears eight tentacles (Figs. 6, 13, and 14). This number is in agreement with the statements of Harmer and Marcus.

The tentacles, when retracted, are pulled into the introverted tentacular sheath in a manner characteristic of the Bryozoa (please compare Figs. 6 and 18).

They surround the entrance to the digestive system which is a U-shaped tract consisting of pharynx, esophagus, proventriculus, stomach and intestine. The most interesting features about the tract are the great length of the esophagus and the presence of a muscular and chitinized proventriculus or gizzard between the stomach and esophagus.

The proventriculi of various species of *Buskia* or *Aeverrillia* are illustrated in papers by Osburn and Veth (1922, Plate I) and Marcus (1941, Plate X, Figs. 44B and 45). Marcus figures the gizzard of both *A. armata* and *A. setigera*. However, the proventriculus of the Massachusetts specimens of *A. setigera* resembles that of his *A. armata* as much as it does that of his *A. setigera*.

The proventriculus of the Massachusetts *A. setigera* is a compact, rounded organ consisting of four conical chitinous sconces capping the internal epithelium. A wide band of circular muscle fibers surrounds these four sconces (Fig. 19). An end view of the proventriculus showing the relation of the four sconces to each other is pictured clearly in Figure 15 and suggested in Figures 9 and 18. A side view, showing the relation of the circular musculature to the sconces and the relative position of the proventriculus in the polypide, is depicted in Figures 13, 14, and 16. A detailed picture of the arrangement of the chitinous and sometimes brown-colored denticles on the sconces appears in Figures 1, 3, 4, 5, and 19. The denticles seem to have a definite arrangement in several roughly V-shaped rows. They are of various sizes. Their color ranges from pale yellow to brown. The shape of each scone at the base ranges from a broad ellipse to a circle. In side view the scone

may appear globose, conical, or even slightly flattened, except for the projecting teeth. Careful inspection of an old or empty colony may occasionally reveal sconces of degenerated polypides still within the otherwise empty zooecia. Because the sconces are usually transparent, pale yellow, and small it is easy to overlook them. In degenerating polypides the gizzard can usually be distinguished as the central part of a dark mass of degenerating material.

The relations of the stomach and intestine to the gizzard and to the lophophore can be seen in Figures 13, 14, and 18. In these three instances the digestive tract is empty. In a mature feeding individual the digestive tract is considerably longer, as a study of Figure 15 will show. The intestine opens outside the circle of tentacles—a characteristic of the Ectoprocta.

The musculature of the lower half of the autozoid was difficult to study partly for lack of sufficient living material and partly because in a mature zoid the digestive tract occupies so much of the interior. However Figure 13 does show a suggestion of a band of retractor muscle fibers attached to the base of the tentacular crown or the upper part of the digestive tract.

The other major muscles attaching to the body wall are the horizontally or circularly arranged parietal muscles. Harmer (1915 p. 88) states that three groups of parietal muscles are visible in his specimens. In the Massachusetts specimens it appears as if there are four groups (Fig. 15).

In a few near-empty zooecia, from which the musculature, tentacles, setigerous collar, and digestive tract were missing but which had a brown body (a mass of dedifferentiating or degenerating tissue) in the upper half of the zooecium, was noticed a rather peculiar globular membranous sac attached to the base of the interior of the autozoid, in the vicinity of the septum which separates the autozoid from the peduncle. This globose mass was hollow. Its wall was soft membranous, and turgid. It is not figured here. Its appearance and position suggest one of two possibilities: 1, it may be a regenerating mass which would give rise to a new polypide within the old zooecium; or 2, it may represent the remains of a degenerating polypide, exclusive of the brown body which was already evident in the upper half of the zooecium. In the fresh water Bryozoa, when polypides of a colony degenerate, sometimes the wall of the colony forms a hollow membranous sac which may either degenerate completely or give rise to a new colony (Rogick, 1938; p. 197).

In studying any form, measurements are extremely helpful. Therefore, as complete a set of measurements of *A. setigera* as was possible was made and is arranged in Table I. The letters and parts A to P are clearly indicated in the drawings of Plates I or II.

#### DISCUSSION

*Aeverillia setigera* seems very widely distributed circumtropically. It has been reported previously from such widely scattered localities as north and east of Australia, China Sea, Gulf of Bengal, Malay Archipelago, Suez Canal, Porto Rico, Brazil's Bay of Santos, etc., whose latitudes range from approximately 24° S to 31° N. The present report extends its range to 41°38' N. Latitude. A recent report (Hutchins, 1945; from Long Island Sound) cites its occurrence slightly south of the present paper. In spite of this extensive range the number of reports

on the occurrence of this species have not been too numerous: Harmer, Hastings, Hincks, Hutchins, Kirkpatrick, Livingstone, Marcus, Osburn, Thornely, and the present writer.

The Massachusetts specimens agree essentially in measurements and appearance with those found in more southerly waters (Gulf of Bengal, South America, and Porto Rico) by previous workers.

Because of their small size and inconspicuous appearance they are easily overlooked when collecting. Very little is known of their behavior, embryology, life history, and physiology. A study should be made of these as well as of colony degeneration, regeneration, rate of growth, development of the proventriculus and setigerous collar, the location and development of the reproductive system, and the nature of the larva. All the work done so far on this form has been of taxonomic nature. The present paper has added a more complete account of the anatomy, included measurements of a number of parts hitherto unmeasured and added a more complete series of diagrams than have existed previously for this species.

#### SUMMARY

1. *Aeoverrillia setigera* was found at Woods Hole and at New Bedford, Mass. This extends its northerly range to 41°38' N. Latitude.
2. The Massachusetts specimens agree closely in appearance and measurements with specimens from more southerly waters of the Gulf of Bengal, Malay Archipelago, South America, and Porto Rico.
3. Measurements of many structures or parts not measured by other workers are here included.
4. The species has been more fully illustrated.
5. The species did not seem to be abundant in the localities from which it has just been reported.

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# STUDIES ON FRESH-WATER BRYOZOA. XVI. FREDERICELLA AUSTRALIENSIS VAR. BROWNI, N. VAR.

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## INTRODUCTION

This study deals with a *Fredericella*, *F. australiensis* Goddard 1909, which was reduced to variety rank and to which were added two other varieties, one of them new. The new variety is here named *F. australiensis* var. *browni*, in honor of Dr. Claudeous J. D. Brown of the Michigan Department of Conservation, Ann Arbor, Michigan, who most generously turned over the material to the author for further study.

The specimens were collected in fair abundance on August 3, 1942, from rocks in an alkali pond about three miles northeast of Church Butte, Uinta County, Wyoming, U.S.A., by Dr. Henry van der Schalie of the University of Michigan, at Ann Arbor.

The writer wishes to express her deep appreciation to both Dr. van der Schalie and Dr. Brown for the opportunity to examine the specimens and to make the present study.

Observations were made on preserved material which was dissected and on preserved material which had to be imbedded and sectioned. No living specimens were available. Dissection and sectioning were necessary to determine tentacle number, diameter of various parts, and internal structure since the zooecial wall was too opaque to permit ready observation of internal structures.

It was necessary to create a new variety, var. *browni*, for the Wyoming form because it resembled very closely in some respects and differed somewhat in other respects from two other forms known heretofore as *Fredericella sultana* subsp. *transcaucasica* Abricossoff 1927 and *Fredericella australiensis* Goddard 1909.

It was necessary to reduce the original *F. australiensis* of Goddard to variety rank, making it *F. australiensis* var. *australiensis* and to add to it two other varieties because the three forms so closely resembled each other and differed noticeably from the long established species of *Fredericella sultana*. Consequently, the former *F. australiensis* Goddard and the *F. sultana* subsp. *transcaucasica* Abricossoff become varieties under the emended *F. australiensis*, namely, *F. australiensis* var. *australiensis* and *F. australiensis* var. *transcaucasica*. The finding of the Wyoming specimens adds a third variety, *browni* to this emended species.

#### FREDERICELLA AUSTRALIENSIS, EMENDED

##### *Description*

The colony is attached along the bases of a number of zooecia whose tips become erect at the distal end and eventually give rise to upright branches which usually do not fuse into a solid mass but which form rather openly branched tufts (Fig. 4). Branching is antler-like or very roughly dichotomous. Septa or dissepiments are absent. Zooecial tubes are slightly wider than those of *F. sultana*. The degree of incrustation of the ectocyst varies from almost none in var. *transcaucasica* to a considerable amount in var. *browni* and var. *australiensis*. Floatoblasts are absent. Sessoblasts are rounded or very broadly elliptical, not reniform or very elongate as those of *F. sultana*. They are shorter and broader than those of *F. sultana*. More exact data or measurements will be given in the "Discussion" section. The terms sessoblasts and floatoblasts have been defined in the author's Study XIV. The *F. australiensis* polypides are shorter and stubbier than those of *F. sultana* and are restricted to the zooecial tips whereas those of the latter species are longer and extended further down into the zooecial tubes. The tentacle number is larger in *F. australiensis* than in *F. sultana*. The former has approximately 24 to 30 tentacles while the latter has about 17 to 24 tentacles. The lophophore is decidedly elliptical in var. *australiensis*. In the other two varieties it is uncertain whether the lophophore is nearly circular or definitely elliptical. Living specimens are necessary to determine this point. However, the lophophore is not horseshoe-shaped, except only in the retracted condition. An epistome is present.

*Fredericella australiensis* is characterized by the rounded, broadly elliptical shape of the sessoblasts, the larger number of tentacles and greater zooecial tube diameter, all admittedly somewhat variable characters but unfortunately almost the only ones, barring nature of colony growth and degree of incrustation which in themselves are variable, on which one can make a distinction in this genus.

*Discussion**Growth habit*

*Fredericella australiensis* and *F. sultana* have a similar growth habit and colonial appearance. The mode of branching is similar. Zoids are adherent for a distance then give off upright branches. Branching is antler-like or very roughly dichotomous in both.

*Dissepiments or septa*

Allman (1856, p. 112) says of *F. sultana*, "At the origin of the branches there is frequently found a more or less perfect septum." His Plate IX, Figure 3, shows an imperfect or partial septum, i.e., a septum with a hole in it. This chitinous septum is located at the commencement of a branch. Kraepelin (1887) calls the dissepiments rudimentary. In *F. australiensis* there seem to be no septa at the start of the branches. Goddard (1909, p. 490) finds none in var. *australiensis*. Abricossoff (1927b, p. 88) shows none in his Figure 2 of *transcaucasica*, and there appear to be none in var. *browni* (present study).

*Keel*

There seems to be relatively little difference between *F. sultana* and *F. australiensis* in this character. The zooecial tubes are cylindrical or nearly so in younger *F. sultana* zooecia and keeled in older specimens, so there occur specimens with and without a keel. This is true also of *F. australiensis*—some individuals may have and others may lack a keel.

*Zooecial tube*

The two species differ very slightly in the shape of the zooecial tubes, when viewed in cross section. The *F. sultana* tubes vary in cross section from cylindrical in unkeeled specimens to somewhat pear-shaped in keeled ones. In *F. australiensis* the tube cross section ranges from an ellipse (in var. *browni*, Figs. 1 and 10) to a rough triangle (var. *australiensis*).

There is a greater difference between the two species in width of zooecial tubes. Those of *F. sultana* are more slender. The diameter of *F. sultana* zooecial tubes of New Rochelle and Lake Erie specimens as given in Study IX (Rogick, 1940, p. 195) ranged from 0.16 to 0.35 mm. and averaged 0.24 mm. for 44 readings. Abricossoff (1927b, p. 91) said that in the U.S.S.R. *Fredericella sultana* the zooecial tube was not more than 0.4 mm. wide. He placed that as the upper limit but did not give the minimum nor average measurements for the point in question. The zooecial tubes of *F. australiensis* are greater in diameter than those of *F. sultana*. Abricossoff (1927b, p. 91) gives the average diameter in *transcaucasica* as 0.5 mm. while the present writer gives a range of 0.259 to 0.576 mm. or an average of 0.391 mm. for the most typical region of a var. *browni* zooecial tube. Thus it would seem that as regards this particular character, var. *browni* is somewhat closer to *F. sultana* than is var. *transcaucasica*.

### *Ectocyst*

There is little difference in appearance between the two species so far as chitinized ectocyst is concerned. In *F. sultana* the degree of incrustation of the ectocyst may vary to such an extent that the zooecial tubes may be translucent to opaque, generally favoring the latter. Debris, stone particles and even algae may attach to it. In *F. australiensis* the degree of incrustation varies also from extremely little in var. *transcaucasica* to the usual "opaque," reasonably well incrustated amount in the other two varieties. Sand grains and debris form part of the incrustation. The color of the ectocyst varies from tan to light brown, in *F. australiensis*.

### *Polypide*

Kraepelin (1887, p. 99) says that polypides of *F. sultana* are very long. Allman (1856, Pl. IX, Fig. 7) shows such a specimen. In samples observed by various workers, including the present one, the polypides of this species seemed long and slender. On the other hand, in *F. australiensis*, the polypides appear distinctly shorter and stubbier, and are restricted to the zooecial tips (see Goddard, 1909, Fig. 12). Since no digestive tract measurements exist for *F. sultana* it is necessary to judge the relative length of its tract by studying Allman's and other workers' drawings. These measurements would vary with the age and condition of nourishment of the polypides.

### *Tentacular crown*

In *F. sultana* the tentacles are long and slender but no measurements exist for them so far as can be determined. In *F. australiensis* the tentacles are generally shorter and stubbier with the possible exception of var. *australiensis*. In the latter variety they measure about one mm. in length and 0.01 mm. in diameter. In var. *browni* the tentacles are shorter and thicker. Unfortunately not too many were in a position to be measured accurately so that one had to depend on the general appearance of those dissected out of the colonies and on a few which were sectioned in the proper plane. These ranged from 0.383 to 0.514 mm. in length and from 0.019 to 0.029 mm. in width (Table II). This is shorter and wider than in var. *australiensis*. No measurements are available for var. *transcaucasica* tentacles. One has to judge them from Abricossoff's (1927b, p. 88, Fig. 2) figure in which they appear shorter and stubbier than tentacles of his *F. sultana* (*ibid.*, Fig. 1).

The number of tentacles does not seem to vary as much in *Fredericella* individuals as it does in those of *Plumatella* and *Hyalinella*. In *Hyalinella punctata*, the author (1945, Study XV, p. 69) found that the ancestrula or first polypide of a colony could be distinguished from successive polypides on the basis of the number of tentacles. It had about  $10 \pm$  less than successive polypides did. Whether the same general principle holds for *Fredericella* and other fresh-water forms could easily enough be determined by germinating statoblasts of the various forms and keeping accurate counts of the number of tentacles developed in each zoid.

The tentacle number of the two species of *Fredericella* is different. In *F. sultana* it ranges from 17 to 24, with 20 to 22 being the most common number. In *F. australiensis* the number ranges from 24 to 30.

TABLE I  
Comparison of the three varieties of *Fredericella australiensis* and including Borg's African specimens

| Part or structure                                       | var. <i>australiensis</i>  | var. <i>browni</i>                             | var. <i>transcaucasica</i>  | Borg's African specimens   |
|---|--|--|---|--|
| A. Lophophore x-section<br>1. expanded?<br>2. retracted | about $0.38 \times 0.23$ mm.                                     | $0.182 \times 0.133$ mm.<br>average            | no data given   | no data given  |
| B. Sessoblast length and width                          | no data given  | $0.382 \times 0.316$ mm.<br>average            | $0.470 \times 0.315$ mm.<br>average                                 | Type A sessoblasts, $0.37-0.43$ mm.<br>long by $0.22-0.27$ mm. wide<br>Type B sessoblasts, $0.33-0.40$ mm.<br>long by $0.30-0.35$ mm. wide |
| C. Zoecial tube diameter                                | no data given  | $0.391$ mm. average                            | $0.5$ mm. average   | Creeping part of tube, $0.33-0.45$ mm.<br>Erect part of tube, $0.24-0.33$ mm.  |
| D. Tentacle number                                      | 28-30  | 24-28  | no data given   | 24-28; usually 26-27   |
| E. Ectocyst appearance                                  | chitinous, brown, incrustated                                    | chitinous, well incrustated, tan, quite opaque | chitinous, thick, light brown, transparent, very little incrustated | chitinous, considerably incrustated with sand grains   |
| F. Zoecial tube in x-section                            | roughly triangular   | elliptical                                     | no data given   | Some strongly keeled. Triangular in attached parts of colony and rounded in erect part of colony   |
| G. Polypides  | "seen only at ends of filaments"—Goddard, so probably were short | short and stubby; at tips of zoecial tubes     | short and stubby  | sometimes arc-shaped when retracted  |
| H. Source of above information                          | Goddard, 1909  | Rogick, present study                          | Abrićosoff, 1927b   | Borg, 1937   |

Previous authors have given ample data on the tentacle number of *F. sultana*. Allman (1856, p. 112) states that this species has about 24 tentacles. His Plate IX, Figure 2, shows 20 to 24 tentacles on various polypides while his Figure 7 (same Plate) shows 25. Nowhere does he call attention to this large number however. Hyatt's (1868, p. 220) *F. regina*, now a synonym for *F. sultana*, had 18 to 22. Kraepelin's (1887, pp. 92, 103) specimens had 20 to 22 as a rule but could also range from 18 to 24. Braem's (1890, p. 11) ranged from 20 to 22, with one specimen being found which had only 17. Toriumi's (1941, pp. 196-197) had 17 to 23. The present writer has found New Rochelle specimens with 24 (1940, Study IX, p. 195), Lake Erie specimens showing the full range of 18 to 24, but usually with 20 to 22 tentacles (1935, Study II, p. 250).

Borg (1937, pp. 272-275) reported the collection of a *F. sultana*, from the Sahara region of Africa, which had 24 to 28 tentacles, wider zooecial tubes than the

#### EXPLANATION OF PLATE I

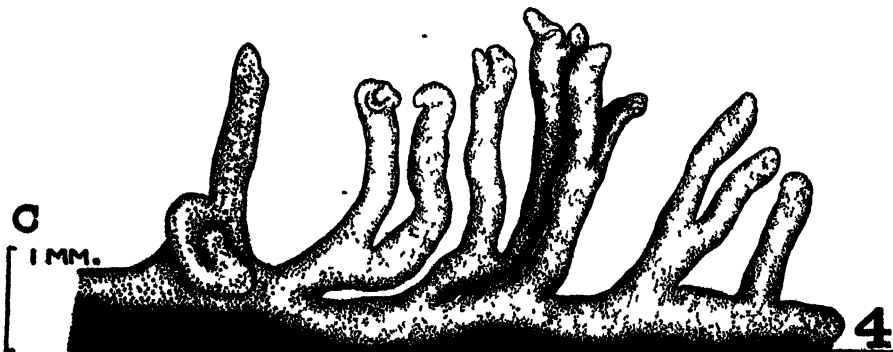
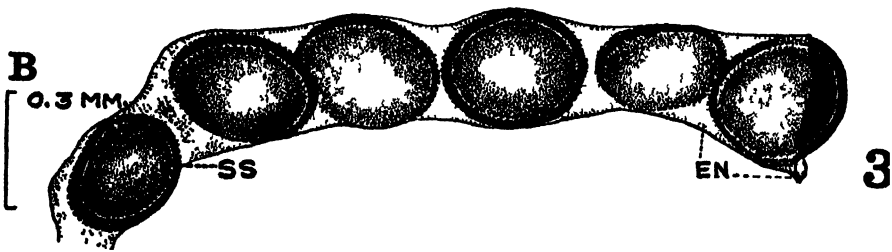
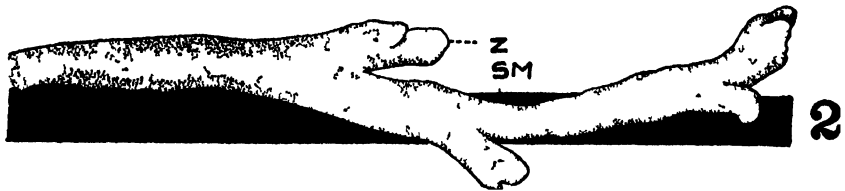
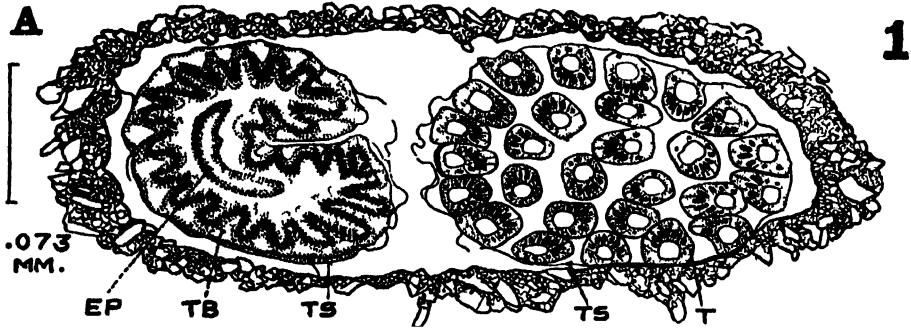
All figures are of *Fredericella australiensis* var. *browni* from the Wyoming collection and have been drawn with the aid of a camera lucida

FIGURE 1. Cross section through a sand and debris incrustated zooecial tube near the tip, which at this level contains the retracted tentacular crowns of two polypides. The tentacular crown at left has been sectioned through the lophophore region at the bases (TB) of the tentacles and through the epistome (EP). The lophophore bears 25 tentacles in this specimen and their bases (TB) at this level are somewhat triangular. The heavy staining of the nuclei accounts for the darkest wavy "stratum" of the tentacles. The lightly stippled material immediately on either side of this dark nuclear "line" or "band" is cytoplasmic material. At this level the surface of the tentacles facing the epistome is ciliated but that is not shown on the drawing. The tentacular crown at left appears to be horseshoe-shaped but that is because it is in the retracted condition. Such a condition also occurs in a retracted *F. sultana* polypide (see Braem, 1890, Pl. V, Fig. 68). The group of 27 tentacles (T) at right belongs to a second polypide. The tentacular cell nuclei are more conspicuous on the inner border of each tentacle where the cells are taller and closer together than on the outer border where the cells are flatter. By inner border is meant the surface facing the epistome and by outer border is meant the surface at the periphery of the tentacular crown. That orientation is best noted in the outermost circle of tentacles. Those within the circle are less regularly oriented. Here again, the cilia have been omitted from the drawing. The zooecial tube is a somewhat longer ellipse here near the zooecial tip than at a level lower down along the tube, as shown in Figure 10. The wall of the tube varies in thickness because of the incrustation. Drawn to Scale A which is 0.073 mm. long.

FIGURE 2. A branch from a colony, showing the zooecial tubes (Z) closely adherent to the substratum (SM), which in this instance is blacked in. The tips of the zooecia are not generally attached to the substratum but are free and directed upward. The condition of the tips indicates that all the polypides are retracted. Drawn to Scale C.

FIGURE 3. Six sessoblasts (SS) shown inside the thin, translucent, tubular, cellular or membranous endocyst (EN). The ectocyst has been removed from the specimen. The cement ring is the darkest part of the sessoblast here. Three of the sessoblasts are turned a little so that one edge shows, but the other does not. The endocyst was torn at the right during dissection and the right-hand statoblast is partly out of it. Drawn to Scale B, which is 0.3 mm. long.

FIGURE 4. Habit sketch of a part of a colony or zoarium showing the adherent base, the upright branches and the mode of dichotomous branching. The substratum is shown in black. When the zooecial tips appear as in this figure their tentacles are either generally retracted or else the tips may be empty. It is sometimes hard to tell if the colony has polypides within it or not because the ectocyst is fairly opaque, so that only very dark structures like the sessoblasts are perceptible with any ease. Since polypide parts are light in color they usually do not show through the ectocyst but have to be dissected out for study. If a colony has been empty a long time the zooecial tips may be broken off and then their emptiness, of course, is evident. Drawn to Scale C which is equivalent to one mm.



ordinary *F. sultana*, and statoblasts which were extremely variable (Table I) and in many cases rounded or oval. Some of his specimens (Borg, 1937, Pl. XVII, Figs. 2 and 3) look very much like *F. sultana* and probably are but his Figure 1 (same plate) appears definitely to belong to *F. australiensis*. Judging by tentacle number, zooecial tube diameter, and appearance of the pictured statoblast inside its tubes, it seems to agree favorably with var. *browni*.

Borg (1937, p. 275) also mentions very incidentally another interesting form of *Fredericella*, *F. sultana* forma *major*, from the north of Sweden, which has 28 to 32 tentacles and is generally of a greater width (presumably zooecial tube width). This would be in conformity with *F. australiensis*. Unfortunately however, he gives no description, pictures, or dimensions of it so that its status is quite uncertain. It may either prove a new species of *Fredericella* or a new variety of *F. australiensis*. At any rate it would be worth a fuller investigation.

#### EXPLANATION OF PLATE II

These are all figures of *F. australiensis* var. *browni* (from the Wyoming locality) and were drawn with the aid of a camera lucida.

FIGURE 5. Surface view of the greater part of one fairly young completed sessoblast. The chitinous substance of the valve gradually thins out toward the center which part is the last to be closed over by the chitin in development. In this specimen the central region was thinnest and palest in color. Drawn to Scale H.

FIGURE 6. An abnormally shaped sessoblast. There were relatively few mis-shapen sessoblasts found in the collection and this was one of them. Its drawing is included as a contrast to the typical sessoblasts shown in Figures 9 and 11. The sessoblast valves are joined together at the border in what is sometimes called a cement ring (CR). The sessoblast contains opaque germinative material (GM) occupying almost all the space between the two capsule valves. The cement ring is dark amber color while the valves are a paler amber.

FIGURE 7. A tentacular crown dissected from a zooecial tube, from preserved material. It shows the relative length of the tentacles. The tentacular mass was slightly disarranged during dissection. Drawn to Scale D whose length is given below the figure.

FIGURE 8. A side or edge view of a sessoblast. The two irregular dark patches (CH) on one valve are chitinous material which grows on some of the sessoblasts, attaching them to the substratum, or to the wall of the colony. A face view of a similar growth is shown in Figure 9. Drawn to the same scale as Figures 9 and 11.

FIGURE 9. A portion of the cellular endocyst tube (EN) enclosing a sessoblast (SS) on which are growing several irregular or crescent-shaped patches of chitin (CH). The sessoblast is typical, normal. Drawn to Scale E.

FIGURE 10. A cross section of a zooecial tube taken about midway between the tip and the base, shown in silhouette. This section is more typical of the elliptical shape of the ectocyst tube than is Figure 1, which was taken near the tip which housed the broadest part of the polypides. The irregularity of the zooecial wall is due to the material incrusting it (see Figs. 1 and 14). Drawn to Scale F.

FIGURE 11. A sessoblast showing the internal germinal mass (GM) shining through the deep amber-colored translucent capsule. The colors of the rest of the sessoblast at the line of junction of the two valves are as follows. The outermost stippled ring is dark reddish amber while the ring shown in black is a very dark brown. These two dark outer bands represent the cement ring area. The shape of the sessoblast is typical for this variety and species. Drawn to Scale E.

FIGURE 12. Surface view of a portion of a sessoblast valve which is older than that portrayed in Figure 5. A delicate raised chitinous tracery, here shown in black, covers it. Drawn to Scale G.

FIGURE 13. Surface view of a portion of still older sessoblast valve than shown in Figure 12. The raised tracery is coarser, darker, and more prominent. Drawn to Scale G.

FIGURE 14. Surface view of ectocyst showing the minute sand grains and other debris imbedded in it. Drawn to Scale H.

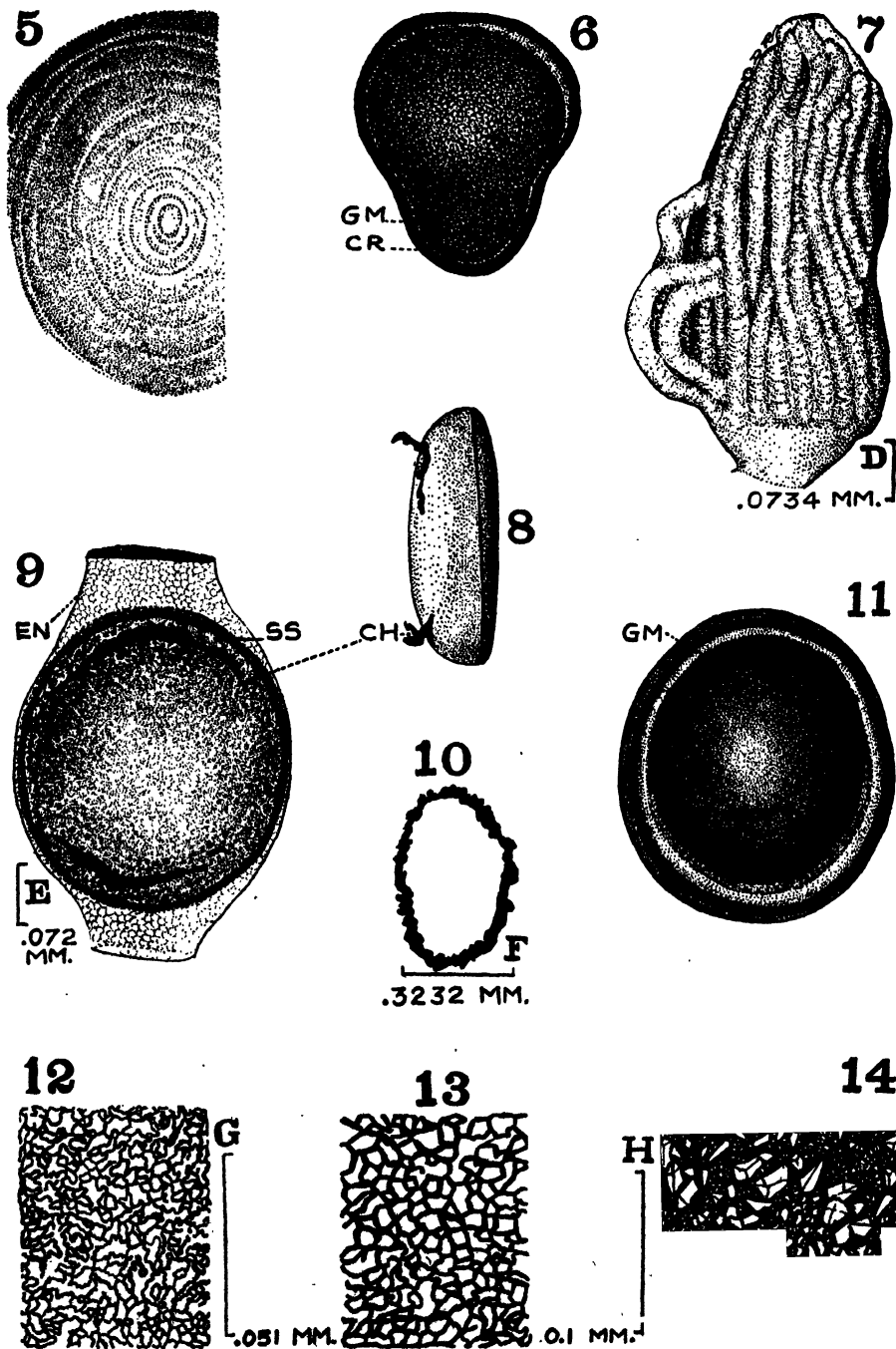


PLATE II

Borg mentions that Kraepelin (1914, reference not available to present author) has collected specimens of *Fredericella* from Rhodesia, Africa, which have statoblasts which are about one third smaller than ordinary German *F. sultana* specimens. Nothing is said about the number of tentacles in the Rhodesian form.

The shape of the expanded tentacular crown in *F. sultana* is nearly circular. In *F. australiensis* var. *australiensis* the lophophore is very definitely elliptical in shape, measuring  $0.23 \times 0.38$  mm. In var. *browni*, it can not be said for certain what the shape is in expanded lophophores since all were retracted in the material studied. Abricossoff makes no mention of this point in var. *transcaucasica*. Cross sections of retracted *F. sultana* and *F. australiensis* look similar except that the latter species has a greater number of tentacles. When the polypides of both species are withdrawn, their lophophores assume a crescent or horseshoe shape (Fig. 1 of present study; Braem, 1890, Pl. V, Fig. 68; Goddard, 1909, p. 491 and Pl. XLVII, Fig. 5).

### Sessoblasts

Statoblasts are extremely important in identification of fresh-water Bryozoa, but those of *Fredericella*, *Plumatella*, and *Hyalinella* often are not entirely adequate in themselves, especially when present in very small numbers, to determine the exact variety or sometimes even the species to which they belong. It is necessary that sufficient specimens be available so that the normal type of statoblast can be observed, for there is so much variation in shape and size that one can readily be misled by examination of just one or two lone statoblasts. There is a great amount of intergradation between statoblasts of different varieties and species. Almost every worker has rather helplessly commented on the fact, yet has been unable to find a criterion that is invariable by which to identify the species and varieties. Statoblasts alone of the above forms are often insufficient for absolute identification. One should also have the colonies and polypides, living and preserved, in sufficient quantity to really make accurate identifications.

In *Fredericella* there is apparently a complete series of intergrading sessoblasts between *F. sultana* and *F. australiensis*. However, the vast majority of the *F. sultana* statoblasts are reniform or quite elongated while the majority of the *F. australiensis* sessoblasts are more rounded or broadly elliptical in outline.

The extreme dimensions for *F. sultana* sessoblasts are: length range from 0.27 to 0.57 mm. and width range from 0.139 to 0.37 mm. The minimal figures above are from some Lake Erie specimens (Rogick, 1935, Study II, p. 250) and the maximal figures are for some European specimens (Kraepelin, 1887, p. 104). As a rule, the average length and width figures show that *F. sultana* sessoblasts are considerably longer than wide, a fact that can not always be fully appreciated from lone maximum and minimum figures. The extreme dimensions, so far determined for *F. australiensis* sessoblasts, are: length range from 0.331 to 0.470 mm., width range from 0.267 to 0.367 mm. if var. *browni* and var. *transcaucasica* (Tables I and II) are considered, or 0.22? to 0.367 if Dr. Borg's African specimens are included in these computations and if the African forms should all prove to belong to *F. australiensis* and not to *F. sultana*. The reason for the question mark after 0.22 in the preceding sentence is that this particular measurement may or may not have been of this species or variety. The average length and width of *F. australiensis* statoblasts,

TABLE II

*Measurements of Fredericella australiensis* var. *browni* from Wyoming

| Part or structure  | Maximum   | Minimum   | Average   | Number of readings |
|--|-----------|-----------|-----------|--------------------|
| A. Sessoblast  |           |           |           |                    |
| 1. Total length  | 0.461 mm. | 0.331 mm. | 0.382 mm. | 69                 |
| 2. Total width   | 0.367 mm. | 0.266 mm. | 0.316 mm. | 69                 |
| 3. Thickness in middle   |           |           | 0.101 mm. | 1                  |
| 4. Cement ring diameter  |           |           | 0.014 mm. | 1                  |
| B. Zooeical tube diameter along the longer of the two transverse axes    | 0.576 mm. | 0.259 mm. | 0.391 mm. | 50                 |
| C. Tentacles   |           |           |           |                    |
| 1. Number  | 28        | 24        | 26-27     | 26                 |
| 2. Length  | 0.514 mm. | 0.383 mm. | 0.451 mm. | 3                  |
| 3. Broadest part of the shorter transverse diameter                      | 0.029 mm. | 0.020 mm. | 0.025 mm. | 10                 |
| 4. Longer transverse diameter (at right angles to preceding measurement) | 0.027 mm. | 0.019 mm. | 0.024 mm. | 14                 |
| D. Lophophore retracted within zooeical tube:                            |           |           |           |                    |
| 1. Antero-posterior diameter   | 0.308 mm. | 0.147 mm. | 0.182 mm. | 8                  |
| 2. Lateral diameter  | 0.170 mm. | 0.111 mm. | 0.133 mm. | 8                  |
| E. Epistome  |           |           |           |                    |
| 1. Antero-posterior diameter   |           |           | 0.019 mm. | 1                  |
| 2. Lateral diameter  |           |           | 0.056 mm. | 1                  |
| F. Esophagus   |           |           |           |                    |
| 1. Length  |           |           | 0.193 mm. | 1                  |
| 2. Width   | 0.060 mm. | 0.051 mm. | 0.054 mm. | 3                  |
| G. Stomach   |           |           |           |                    |
| 1. Length  | 0.653 mm. | 0.634 mm. | 0.644 mm. | 2                  |
| 2. Width   | 0.070 mm. | 0.066 mm. | 0.068 mm. | 2                  |

at least of the *browni* variety, show that the statoblasts are more nearly a broad ellipse than are those of *F. sultana*. The *F. australiensis* sessoblasts are generally slightly flattened on one side and very probably roughened by various markings on the other, when mature (Figs. 12 and 13). Neither Goddard nor Abricossoff mention the nature or pattern of the surface markings on their specimens' sessoblasts. Variety *browni* however had some sessoblasts with markings (Figs. 12 and 13); so does *F. sultana* (Rogick, 1937, p. 102, Fig. 1).

### Distribution

*Fredericella australiensis* has a widely scattered distribution although it has been reported relatively few times. Its three varieties are distributed as follows. Variety *australiensis* occurs in the water supply system at Pott's Hill in New South Wales, Australia (Goddard 1909, pp. 487-489). Goddard reported that the *F.*

*sultana* recorded earlier from Australia by Whitelegge is probably his own *F. australiensis*. Variety *transcaucasica* occurs in Lake Madatapeen, Tiflis District, the Transcaucasus, in the U.S.S.R. (Abricossoff 1927a, p. 308 and 1927b, p. 91). This variety was collected by B. S. Winograd on July 1, 1915 and later identified by Dr. Abricossoff. Variety *browni* occurs in Uinta County, Wyoming, U.S.A. Some of Dr. Borg's material from rivers in the Sahara region of North Africa is very likely *F. australiensis* var. *browni*. This widens the distribution of *F. australiensis* to 4 ? continents: Africa?, Australia, Eurasia, and North America.

#### Key to Varieties of *Fredericella australiensis*

- 1 (2) Chitinous ectocyst well incrustated with sand grains and debris; rather opaque.....3
- 2 (1) Chitinous ectocyst very little incrustated; very transparent; zooecia about 0.5 mm. wide; sessoblasts average  $0.315 \times 0.47$  mm.....var. *transcaucasica*
- 3 (4) Tentacle number 24-28; sessoblast average  $0.316 \times 0.382$  mm.; zooecial tubes elliptical in cross section.....var. *browni*
- 4 (3) Tentacle number 28-30; zooecial tubes roughly triangular in cross section  
var. *australiensis*

#### FREDERICELLA AUSTRALIENSIS VAR. BROWNI, NEW VARIETY

##### \*Description and Discussion

This variety is illustrated in Figures 1 through 14. Its measurements are given in Table II. Its points of difference and resemblance as compared with the other two varieties are briefly summed up in Table I. Some gaps exist in the information about this variety and they are: 1, the shape and dimensions of the expanded lophophore and 2, the unavailability of living specimens for a more complete study of tentacle and polypide size and variation. However, on the basis of the preserved material available, the following description of the variety can be made.

Variety *browni* has a thin chitinous ectocyst well incrustated with sand grains and debris (Figs. 1 and 14). It is of light tan color and rather opaque. The opacity of the zooecia is such that it is possible to see whether the much darker colored sessoblasts are present, but not whether polypides are present because the light color of the polypides blends in so well with the color of the incrustated ectocyst. To determine if tubes contain polypides it is frequently necessary to tear them apart. Only then are the polypides visible.

Basal zooecia are recumbent or adherent in their more proximal part, with the tips directed upwards (Figs. 2 and 4). From these arise erect branches (Fig. 4). The zooecia are generally elliptical in outline (Figs. 1 and 10). Occasionally a faint keel may be present (Fig. 2) but usually it is not noticeable. The colony appears upon rocks as a coarse tracery or tufted mass, depending upon the number of polypides in it. If the number of polypides is small or if the periphery of the colony is examined there will be located the more adherent members. If the colony is luxuriantly branched and on a rather limited substratum then it has many more upright branches. These are not fused together but retain their individuality and open mode of branching. The zooecia are usually very long (Fig. 4). The ectocyst has considerable rigidity and firmness. The zooecia are somewhat wider than in *F. sultana*. Those of var. *browni* are not as wide apparently as those of var. *transcaucasica* (Table I). The ectocyst is too opaque to be able to see dissepiments or incomplete septa at the commencement of the zooids even if they

were present in this variety. Such dissepiments occur in *F. sultana*. A diligent search was made through sectioned and dissected *F. australiensis* var. *browni* material but no dissepiments could be found.

The ectocyst is lined with a soft thin transparent membranous endocyst. The endocyst encloses the polypides and sessoblasts (Figs. 3 and 9).

The polypides of var. *browni* appear short and stubby. The tentacles, especially, seem so, perhaps because of their considerable number, 24–28 (Fig. 7). The tentacles ranged in number from 24 to 28 but the usual number was 26 or 27, just as Borg had found in his African specimens. Of course, the condition of the colony, the length of the polypides and tentacles are greatly influenced by the state of nutrition of the colony. The better fed the colony, the longer the polypides and tentacles. However, the var. *browni* specimens seemed well enough nourished. Their digestive tracts were well filled with algal food.

The parts of the digestive tract are the same as for *F. sultana* and *Plumatella repens*—ciliated mouth guarded by the epistome, ciliated pharynx, esophagus, stomach, and intestine.

The reproductive organs were not observed.

The sessoblasts of var. *browni* are generally smooth on one side (Fig. 5) and roughened on the other (Figs. 12 and 13). However, some older sessoblasts may show roughening or markings on both sides, and in addition, chitinous material may begin to grow on the valve of the statoblast (Fig. 8), attaching it to the endocyst (Fig. 9) or to the body wall and possibly eventually to the substratum.

Variety *browni*'s sessoblast shape is best shown in Figures 9 and 11, which are typical. Abnormal specimens occasionally occur and one such is shown for contrast in Figure 6.

The colors of the sessoblasts range from reddish yellow to brown, depending upon the age; the older, the darker.

There were quite a number of sessoblasts present in the zooecial tubes of the Wyoming specimens at the time of collection (August).

The sessoblasts were so distinctive in shape and general proportions that it was immediately evident that one was not dealing with *F. sultana* but with a form related to Abricossoff's and Goddard's specimens—a distinct species—*F. australiensis*.

The decision to make each of these forms (*F. australiensis*, *F. sultana transcaucasica*, and the Wyoming specimens) a separate variety of *F. australiensis* was based on the great similarity to each other so far as the shape of their statoblasts was concerned and their slight but distinct differences as regards the nature of the ectocyst and the number of tentacles (refer to Key to Varieties and Tables I and II).

#### SUMMARY

1. The species *Fredericella australiensis* has been emended to include three varieties.
2. A new variety, *F. australiensis* var. *browni*, has been erected.
3. Two other previously recorded forms, *F. australiensis* Goddard 1909 and *F. sultana* subsp. *transcaucasica* Abricossoff 1927 have been reduced to the status of varieties under the emended *F. australiensis*.
4. The emended *F. australiensis* is characterized by its rounded or broadly

elliptical sessoblasts, its wider zooecial tubes, its greater tentacle number, its lack of dissepiments and the shorter stubbier tentacles and polypides which are generally confined to the tips of the tubes. These features distinguish it from *F. sultana*.

5. The varieties *australiensis*, *browni*, and *transcaucasica* are placed in *F. australiensis* because they possess the above characteristics.

6. The three varieties are distinguished from each other on the basis of degree of incrustation of their ectocyst, the difference in number of tentacles, appearance of the zooecial tubes in cross section and miscellaneous measurements.

7. *Fredericella australiensis* has a wide but scattered distribution. It is represented in Australia by var. *australiensis*; in Eurasia (the U.S.S.R.), by var. *transcaucasica*, in Africa?; and in North America, by var. *browni*.

8. The specimens which were immediately responsible for the erection of the new variety, *F. australiensis* var. *browni*, were obtained through the kindness of Dr. C. J. D. Brown and Dr. H. van der Schalie of Ann Arbor, Michigan, who turned the collection over to the author for study. The specimens were collected by Dr. van der Schalie on August 3, 1942, from rocks in an alkali pond about three miles northeast of Church Butte, Uinta County, Wyoming, U.S.A.

9. The study includes 14 illustrations and one table of measurements dealing with var. *browni* and one table of comparison between the three varieties.

10. A brief summary of available measurements and other data on *F. sultana* is given.

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## STUDIES ON THE BIOCHEMISTRY OF TETRAHYMENA. VII. RIBOFLAVIN, PANTOTHEN, BIOTIN, NIACIN AND PYRIDOXINE IN THE GROWTH OF *T. GELEII* W

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With the substitution of chemically known materials for all but one fraction in the medium for the growth of *Tetrahymena* it has been possible to determine with some degree of exactness the specific vitamin requirements of this important ciliate. When proteins, such as casein or gelatin, or peptones are used as the base medium it has been impossible to determine the importance of those vitamins which were stable to treatments which would not also destroy other essential materials. Using these types of media, claims have been made for the essential nature of thiamine and of riboflavin for *Tetrahymena geleii* (Hall and Cosgrove, 1944; Hall, 1944). It was earlier indicated (Kidder and Dewey, 1942) and later conclusively proven (Kidder and Dewey, 1944; 1945a; 1945b) that at least eight strains of *Tetrahymena* could grow in a medium in which the thiamine had been destroyed.

When it was found that *T. geleii* could be grown successfully in a mixture of amino acids (Kidder and Dewey, 1945c) and that two of the three "unknown growth factors" could be replaced with nucleic acid derivatives (Kidder and Dewey, 1945d) and that the remaining "unknown growth factor" (Factor II) was relatively stable and was not adsorbed readily on activated charcoal, it became possible to examine the effects of the omission of a number of the vitamins. Hitherto these vitamins had been added routinely to guard against the possibility of any one of them proving to be a limiting factor. It was found (Kidder, 1945) that folic acid is an essential growth factor for *T. geleii* W, this fact being obscured previously by the necessary inclusion of Factor I (containing folic acid) as the lead acetate precipitate fractions of raw materials, the Factor I activity being readily absorbable on activated charcoal.

The present work has been made possible by the utilization of a number of different treatments of the Factor II preparations and the inclusion of all other constituents of the medium as chemically pure materials. Furthermore, this work would not have been possible without the employment of a microbiological method for the detection of traces of the growth factors under consideration. We have utilized *Lactobacillus casei* as a tool in this study, and while we have made no attempts to assay various preparations quantitatively, we have used the bacterial method for determining the total lack of the vitamin under immediate consideration. It has been possible also, to show that the ciliate possesses the ability to synthesize certain of the B vitamins, by determining the increase of the vitamin by the *L. casei* test after the growth of the ciliate.

<sup>1</sup> Aided by grants from the Morgan Edwards Fellowship Fund and the Manufacturers Research Fund for Bacteriology and Protozoology of Brown University. Present address Biological Laboratory, Stanford University.

## MATERIALS AND METHODS

*Organisms*

The ciliate used in this study was *Tetrahymena geleii* W, which has been maintained in pure (bacteria-free) culture in this laboratory for a number of years and which has been used in numerous previous studies (Kidder and Dewey, 1942–1945). The organism has been grown in amino acid media for the past one and one-half years and all inocula for the present series were taken from these stocks.

*Lactobacillus casei* 912 was used for the microbiological testing of experimental media. This organism was obtained from the Squibb Institute for Medical Research through the courtesy of Dr. Vincent Groupé. Stocks were carried in yeast extract-dextrose-agar stab cultures, transplants being made at monthly intervals, incubated at 37° C. for 24 hours and then placed in the refrigerator.

*Ciliate base medium*

One type of base medium was used routinely. This appears in Table I with the complete supplements. Each vitamin under investigation was omitted from the medium separately.

*Preparation of Factor II*

It has been necessary to treat the Factor II preparations in various appropriate ways in order to eliminate the different vitamins studied. In the earlier work (Kidder and Dewey, 1945d) the prime consideration in the Factor II preparation was the elimination of Factors I and III activity, and the methods used did not necessarily render the preparation vitamin free. In this study the inclusion of Factor I and Factor III activity was of no particular importance, and so attempts were made to eliminate the vitamin under consideration and still retain maximum Factor II activity. This latter was not always possible as some of the treatments used not only removed or destroyed the vitamin but also lowered the Factor II activity. Nevertheless preparations which were satisfactory for this study were obtained, and these will be described under the heading of each vitamin.

*Riboflavin-free preparation (8L531).*

Liver Fraction L<sup>2</sup> (50 grams) was dissolved in one liter of distilled water and a 40 per cent solution of normal lead acetate was added until no more precipitate formed. The precipitate was removed by filtration with the aid of Celite and discarded. The filtrate was neutralized with NaOH and treated with an excess of basic lead acetate. The second precipitate was removed and discarded, the excess lead removed with 9 per cent oxalic acid and the excess oxalic removed as the oxalate with Ca(OH)<sub>2</sub>. Tests at this stage showed the presence of large amounts of riboflavin, but after adsorption with 10 grams of Norit A for one hour at room temperature at pH 3.5 the riboflavin had been quantitatively removed. This preparation was used in a concentration of one part in ten parts of final medium.

<sup>2</sup> Furnished through the courtesy of Dr. David Klein and the Wilson Laboratories.

TABLE I  
Base Medium

|   | mg./ml  |  | micrograms/ml. |
|---|---------|--|----------------|
| <i>l</i> (+)-arginine mono-hydrochloride  | 0.82    | biotin methyl ester <sup>3</sup>           | 0.00005        |
| <i>l</i> (-)-histidine mono-hydrochloride | 0.10    | calcium pantothenate <sup>3</sup>          | 0.10           |
| <i>dl</i> -isoleucine                     | 0.35    | thiamine hydrochloride                     | 0.10           |
| <i>dl</i> -leucine                        | 0.35    | nicotinamide <sup>3</sup>                  | 0.10           |
| <i>dl</i> -lysine                         | 0.60    | riboflavin <sup>3</sup>                    | 0.10           |
| <i>dl</i> -methionine                     | 0.34    | pyridoxine hydrochloride <sup>3</sup>      | 0.10           |
| <i>dl</i> -phenylalanine                  | 0.14    | <i>p</i> -aminobenzoic acid                | 0.10           |
| <i>dl</i> -serine                         | 0.04    | <i>i</i> -inositol                         | 1.00           |
| <i>dl</i> -threonine                      | 0.20    | choline chloride                           | 1.00           |
| <i>l</i> (-)-tryptophane                  | 0.10    | folic acid concentrate <sup>4</sup>        | 1.00           |
| <i>dl</i> -valine                         | 0.20    |  |                |
| dextrose                                  | 2.00    |  | mg./ml.        |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O      | 0.10    | hydrolyzed yeast nucleic acid <sup>5</sup> | 0.05           |
| K <sub>2</sub> HPO <sub>4</sub>           | 0.10    |  |                |
| CaCl <sub>2</sub> ·2 H <sub>2</sub> O     | 0.05    | Factor II preparation (see text)           |                |
| FeCl <sub>3</sub> ·6 H <sub>2</sub> O     | 0.00125 |  |                |
| MnCl <sub>2</sub> ·4 H <sub>2</sub> O     | 0.00005 |  |                |
| ZnCl <sub>2</sub>                         | 0.00005 |  |                |

*Pantothen-free preparation (8L531H).*

Although pantothenic acid is adsorbed on activated charcoal the time and temperature allowed in preparing the riboflavin-free medium is insufficient for the complete removal of pantothen. Raising the temperature, increasing the time, or increasing the amount of Norit used was not practical as the Factor II activity was greatly reduced (Kidder and Dewey, 1945d). Therefore advantage was taken of the sensitivity of pantothenic acid to alkali and heat and the riboflavin-free preparation was adjusted to pH 10.0 with NaOH and autoclaved for two hours. The Factor II activity was somewhat reduced by this treatment, but the preparation was entirely satisfactory for use. *L. casei* tests showed that the pantothenic acid content had been lowered to an insignificant amount. This preparation was used in concentrations of one part in ten parts of final medium.

*Biotin-free preparation (8L5C1).*

The most active biotin-free preparation, and therefore the one used in this study, was made in the following manner. Ten grams of Liver Fraction L was dissolved in 200 ml. of distilled water and brought to boiling. To this boiling mixture were added 10 ml. of a 10 per cent solution of NaHSO<sub>3</sub> and 10 ml. of a 10 per cent solution of CuSO<sub>4</sub>, and boiling was continued for 3–5 minutes. The precipitate was removed on a fluted filter and the process repeated once. The copper was removed as CuS after treating with 15 per cent Na<sub>2</sub>S and the sulfate and sulfite removed as the barium salts after treatment with Ba(OH)<sub>2</sub>. The volume of the filtrate was reduced to 200 ml. and a 100 ml. aliquot was adjusted to pH 3.5. Two grams

<sup>3</sup> Omitted singly in the appropriate series of experiments.

<sup>4</sup> The folic acid concentrate used had a potency of 5000 and was furnished through the courtesy of Dr. R. J. Williams.

<sup>5</sup> Assays of the hydrolyzed yeast nucleic acid with *L. casei* showed it to be free of riboflavin, pantothen, biotin, niacin, and pyridoxine but appreciable amounts of folic acid were present.

of Norit A was added and adsorption allowed to continue for one hour at room temperature with constant stirring. This preparation was used in a concentration of one part in twenty parts of final medium.

#### *Niacin-free preparation (8L5C2)*

The use of copper precipitation, described above, was designed for the removal of nicotinic acid. While most of the niacin activity was removed by this method, as shown by the *L. casei* test, enough remained to warrant further treatment. Accordingly the filtrate from the copper precipitation was extracted with *n*-butanol for 96 hours in a continuous extraction apparatus (Wilson, Grauer, and Saier, 1940). It is known that nicotinamide is readily extracted with butanol, and after this treatment the extract was found to be entirely devoid of niacin activity, even when tested with *L. casei* in amounts four times greater than those used as a supplement for the ciliate. This preparation was used in a concentration of one part in twenty parts of final medium.

#### *Pyridoxine-free preparation (8L531L)*

This preparation was the least successful of any used. While it was possible to treat crude extracts and various filtrates in ways which would remove all pyridoxine activity for *L. casei*, it was usually found that the Factor II activity was also lowered to a point where the preparation was very inferior as a ciliate supplement. Therefore, the most satisfactory preparation, and the one finally used, was very low in Factor II activity, and the results obtained cannot be compared directly with those of the other vitamins tested. This preparation was made by exposing an alkaline lead acetate filtrate fraction (8L531), to direct illumination from a 300 watt electric bulb at a distance of 8 inches for a period of 72 hours. This method was used by Hochberg et al (1943) for pyridoxine destruction. Besides the destruction of appreciable amounts of the Factor II, another disadvantage of the technique was the excessive evaporation which took place during the treatment. It was necessary to add distilled water at frequent intervals to prevent the preparation from drying down. This preparation was used in a concentration of one part in ten parts of final medium.

#### *Assay procedure*

The base medium employed for the testing of the various preparations was the 16 amino acid mixture suggested by Hutchings and Peterson (1943). This was chosen in preference to the casein hydrolysate medium of Landy and Dicken (1942) because of the known composition of the former and the fact that lower blanks can be obtained. While the amino acid medium does not permit the production of as much acid by the bacteria it is very satisfactory for determining the presence or absence of a known vitamin.

Because of the scarcity of amino acids we have modified the usual procedure. The amino acid medium is made up for stock in double strength and the sugar, acetate, salts, purines and pyrimidine are added in double strength. For a test, this complete base medium is measured into 125 × 7 mm. Pyrex tubes in one ml. volumes. The material to be tested is added in appropriate amounts and a mixture

of the vitamins, minus the one for which the preparation is being tested, is added. The volume is then made up to 2 ml. with distilled water. Two controls were run with each test, one containing base medium and a complete set of supplements except for the vitamin under test. The second control contained the base medium plus the complete supplement and plus the Factor II preparation. The first served as a control on carry-over growth. The second was a control on the possible toxicity of the Factor II preparation. When titrations were made the figure from the first control was subtracted from the figure from the unknown preparation. Inasmuch as a small volume of medium was used it was found advantageous and more accurate to reduce the standard hydroxide to 0.05 N. The NaOH was standardized with 0.05 N oxalic acid, and the amount of acid produced after 96 hours of growth at 37° C. was titrated directly, using brom thymol blue as an indicator. The longer incubation period was used for maximum acid production, for in this way the test becomes more sensitive for traces of vitamins.

After many trials, the usual drop method of inoculation of *L. casei* was abandoned in favor of inoculating with a straight needle. This eliminates the necessity for washing the bacteria and blanks are just as low. The inocula were always made from yeast extract cultures which had incubated for 18–24 hours at 37° C.

While standard curves, using this method, have been made for all the vitamins studied the results obtained with our preparations do not permit quantitative statements as to amounts inasmuch as the tests were always made at very high levels and stimulatory materials in the Factor II preparations were invariably present. We were interested, moreover, first in the determination of the vitamin-free condition of our media, and second, in the biosynthesis of the vitamins by the ciliates. In the latter case, assays were employed on the medium before and after ciliate growth and the difference of acid production between the two compared directly.

It has been pointed out (see Cheldelin et al, 1942) that many of the B vitamins occur in a bound form in tissues and must be liberated by some means for satisfactory tests. There was the possibility that bound vitamins in the Factor II preparations might be available for the ciliate but not for *L. casei*, and these would invalidate any conclusions which were based on the vitamin-free nature of the preparation by the *L. casei* test. Enzymatic digestion was carried out on all the preparations, therefore, in order to test for the total vitamin content. Accordingly takadiastase and pepsin in quantities of one per cent each of the total solids of the preparation to be tested were used. The preparation was allowed to digest under toluene for 24 hours at 37° C. at pH 3.5. After steaming, the digest was added to the assay tubes, as described above, and a control of equivalent amounts of the enzymes added to parallel tubes. This latter control is obviously necessary as the enzymes are not vitamin-free. Data on the results of assays of the Factor II preparations used are presented in Table II.

### *Ciliate cultures*

It was the usual practice, when testing for the effects of one of the known vitamins, to grow the ciliate through at least three serial tube transplants in the medium containing the vitamin being investigated, paralleled with the same medium minus the vitamin. Transplants were made at 72 hour intervals with a bacteriological loop delivering approximately 0.005 ml. of fluid. All incubation was at

25° C. Growth rate was followed by inoculating appropriate amounts of third transplant ciliates (36 hours old) into like media in culture flasks (Kidder, 1941). After inoculation of the flasks (as near 100/ml. as possible) samples were drawn and the initial inoculum determined. Growth thereafter was ascertained by sampling at intervals until the termination of the experiment. In all cases the flask series were repeated at least once and the figures averaged.

TABLE II  
*Assay of Factor II Preparations with Lactobacillus casei 912*

| No. | Additions          | Vitamin omitted from base medium |           |        |              |            |            |
|-----|--------------------|----------------------------------|-----------|--------|--------------|------------|------------|
|     |                    | Riboflavin                       | Pantothen | Biotin | Nicotinamide | Pyridoxine | Folic acid |
| 1   | None               | 0.05                             | 0.00      | 0.05   | 0.49         | 0.12       | 0.62       |
| 2   | Enzyme preparation | 0.13                             | 0.38      | 0.17   | 0.75         | 0.10       | 0.90       |
| 3   | 8L531              | 0.05                             | 3.79      | 2.88   | 4.72         | 3.86       | 0.00       |
| 4   | 8L531H             | 0.03                             | 0.12      | 2.79   | 4.80         | 2.60       | 0.00       |
| 5   | 8L5C1              | 0.00                             | 1.53      | 0.07   | 0.22         | 3.10       | 4.36       |
| 6   | 8L5C2              | 3.42                             | 0.87      | 2.93   | 0.00         | 3.65       | 4.65       |
| 7   | 8L531L             | 0.00                             | 3.74      | 3.00   | 4.51         | 0.09       | 0.00       |

Figures represent ml. of 0.05 N acid per culture (2 ml.). All figures corrected for uninoculated blanks. Line 2 corrected for carry-over growth (Line 1). Lines 3-7 corrected for vitamin content of enzyme preparation (Line 2).

One obvious objection to the flask technique is the possibility of introducing the vitamin being investigated from the rubber vaccine tip used in the sampling port. This possibility was diminished by boiling the vaccine tips for one hour previous to setting up the flasks. As a check on the tips uninoculated flasks were manipulated in the same manner as the experimental cultures and the samples tested with *L. casei* for the vitamin being studied. In no case were these detectable amounts of the vitamins present. Sampling needles were made chemically clean as well as sterile before use.

Growth rate during the logarithmic phase was calculated by the formula  $g = \frac{t \log 2}{\log b - \log a}$  where  $t$  = the time in hours during which the population has been increasing exponentially,  $a$  = the number of cells per unit volume at the beginning, and  $b$  = the number of cells at the end of time,  $t$ .

## RESULTS

After obtaining Factor II preparations which were free of the vitamins to be studied, preliminary experiments were set up to determine which vitamins, if any, were essential growth factors for *Tetrahymena geleii* W. Accordingly serial transplants were made in the appropriate media, one set with the vitamin present, and the other with the vitamin omitted. It was immediately apparent that the ciliate lacked all ability to synthesize folic acid (Kidder, 1945) but the absence of none of the other vitamins did more than lower the growth rate and the yield. Growth in

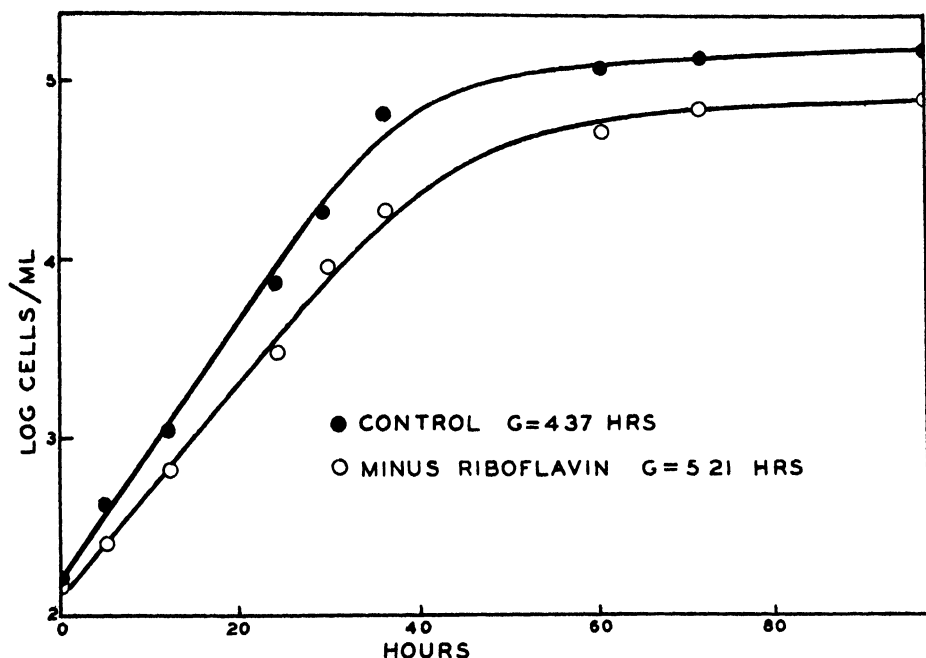


FIGURE 1 Effect of the omission of riboflavin Factor II preparation used was 8L531  
Average of two separate experiments

TABLE III  
*Summary of Growth Data*

| Medium             | Generation time in hours | Population per ml at end of log phase | Population per ml at 96 hours |
|--------------------|--------------------------|---------------------------------------|-------------------------------|
| Control            | 4.37                     | 58,000                                | 164,000                       |
| Minus riboflavin   | 5.21                     | 19,000                                | 67,000                        |
| Control            | 4.57                     | 32,000                                | 90,000                        |
| Minus pantothen    | 4.60                     | 34,500                                | 41,000                        |
| Control            | 4.32                     | 45,500                                | 152,000                       |
| Minus biotin       | 5.01                     | 15,000                                | 96,000                        |
| Control            | 4.17                     | 49,000                                | 170,000                       |
| Minus nicotinamide | 8.40                     | 7,500                                 | 79,000                        |

the sixth serial transplant was possible for all series except that lacking exogenous folic acid.

In order to gain quantitative information regarding the stimulatory effect that was apparent in the tube cultures, growth flasks were inoculated from third transplant tubes and the growth followed by frequent sampling. In the case of pyridoxine, however, the flask cultures were omitted, as the Factor II preparation necessarily used was relatively inactive and the growth was erratic, even when pyridoxine was

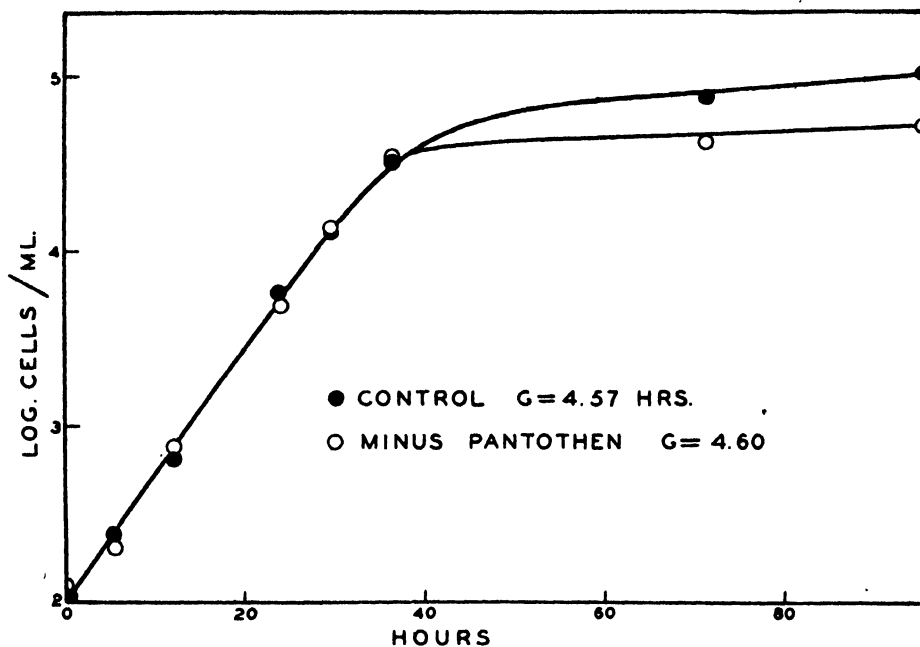


FIGURE 2. Effect of the omission of pantothenic acid. Factor II preparation used with 8L531L. Average of two experiments.

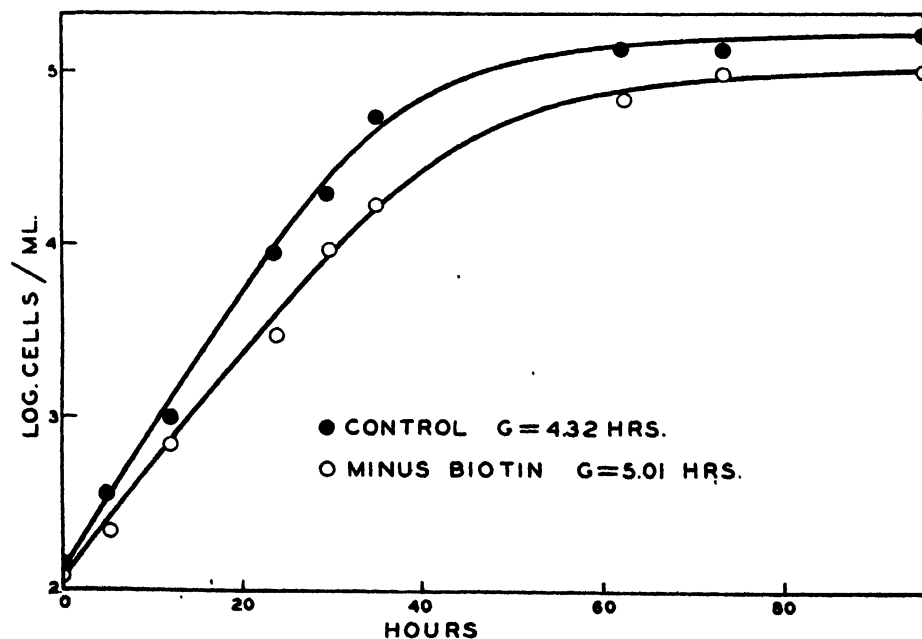


FIGURE 3. Effect of the omission of biotin. Factor II preparation used was 8L5C1. Average of two separate experiments.

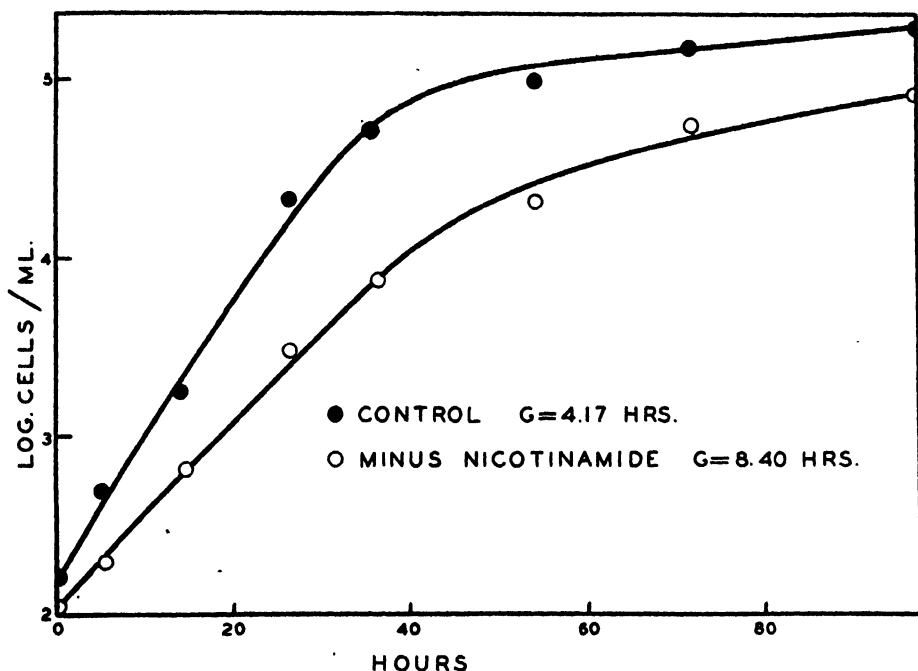


FIGURE 4. Effect of the omission of nicotinamide. Factor II preparation used was 8L5C2. Average of three separate experiments.

present. While qualitative data are lacking for the pyridixine series, nevertheless we can say from the serial tube transplants that this vitamin appears to be only stimulatory for *T. geleii* W.

The omission of riboflavin from the medium resulted in slower growth during the exponential period. Thus the generation time was raised from 4.47 hours in the control flasks to 5.21 hours. The maximum yields were reduced to less than half of those in the control flasks (Fig. 1; Table III).

The ciliates appear to synthesize pantothen at a rate which equals the demands for rapid growth, as judged by the almost identical growth rates in the pantothen-containing and the pantothen-free media (Fig. 2; Table III). In all cases, however, the maximum yield was significantly lower in the pantothen-free cultures.

A comparison of the growth curves, generation times and yields for biotin-free and riboflavin-free media (Figs. 1, 3; Table III) shows remarkable similarity. The rate of synthesis of biotin by the ciliates appears to be low, indicating the stimulatory status of this vitamin. We possess added data on biotin substantiating its non-essential nature for *T. geleii* W. Early in this series of investigations the effect of raw egg white and avidin concentrates were studied as a means of determining whether or not the ciliate required biotin. Egg white was taken aseptically and added to tubes containing 5 ml. of one per cent proteose-peptone, each tube receiving 0.1 ml. According to Eakin, Snell, and Williams (1941), this amount of egg white is enough to inactivate 0.05 micrograms of biotin. The analysis of proteose-peptone made by Stokes, Gunness, and Foster (1944) shows that one gram contains

0.2 micrograms of biotin, hence our tubes each contained 0.01 micrograms of the vitamin. The amount of raw egg white used, therefore, was enough to inactivate five times more biotin than was present. Indefinitely transplantable growth occurred in the proteose-peptone plus egg white. Likewise, the use of avidin concentrates in quantities far in excess of that needed to inactivate all of the biotin present, produced similar results. In this case the avidin was allowed to act on the proteose-peptone, the peptone removed as the diffusate in dialysis, the peptone being used as the medium. Similar results were obtained with proteose-peptone treated with  $H_2O_2$  in a manner similar to that described by Garnjobst, Tatum, and Taylor (1943). While it is clear that biotin is not required by *T. geleii* W this vitamin is stimulatory.

TABLE IV

*Assay Data (L. casei) Before and After the Growth of T. Geleii W*

| Additions for assay (1:10)            | Factor II preparation |                   |                 |                  |             |              |                    |                     |                  |                   |
|---------------------------------------|-----------------------|-------------------|-----------------|------------------|-------------|--------------|--------------------|---------------------|------------------|-------------------|
|                                       | 8L531                 |                   | 8L531H          |                  | 8L5C1       |              | 8L5C2              |                     | 8L531L           |                   |
|                                       | Plus ribo-flavin      | Minus ribo-flavin | Plus panto-then | Minus panto-then | Plus biotin | Minus biotin | Plus nicotin-amide | Minus nicotin-amide | Plus pyri-doxine | Minus pyri-doxine |
| Before inoculation                    | 4.78                  | 0.07              | 3.90            | 0.17             | 3.92        | 0.08         | 3.88               | 0.00                | 3.61             | 0.16              |
| After 72 hr. ciliate growth.          | 4.60                  | 1.18              | 3.94            | 1.71             | 3.90        | 1.56         | 3.80               | 0.21                | 3.48             | 0.22              |
| Medium plus cells                     |                       |                   |                 |                  |             |              |                    |                     |                  |                   |
| Supernatant of 72 hr. ciliate culture | 4.82                  | 0.06              | 4.13            | 0.10             | 3.86        | 0.10         | 3.71               | 0.16                | 3.52             | 0.11              |
| Washed ciliates from 72 hr. culture   | 4.80                  | 1.07              | 3.94            | 1.64             | 3.91        | 1.05         | 3.75               | 0.25                | 3.61             | 0.15              |

Figures represent ml. of 0.05 N acid per culture (2 ml.). All figures corrected for uninoculated blanks and for carry-over growth.

While *T. geleii* W can be transplanted indefinitely in the absence of exogenous nictotinamide this vitamin (or nicotinic acid) is an active stimulant. The generation time is doubled when the ciliate is grown in niacin-free medium as compared to that in the nicotinamide-containing control (Fig. 4; Table III). While the population density at 96 hours is less than one-half that of the control (which is similar to the cases of riboflavin, biotin, and pantothen), the population at the end of the logarithmic phase is extremely low (approximately 7000/ml.).

It was of interest to determine whether or not *T. geleii* W would synthesize amounts of the vitamins which could be detected with the assay methods used. Accordingly the five types of media used above were set up for serial transplants and an aliquot of each was assayed with *L. casei*. After the ciliates had grown for 72 hours in the third transplants, assays were again made for the various vitamins. These assays were of three different types. One was on the whole medium (medium plus cells); one, on the supernatant fluid following centrifugation after chilling (Kidder, Stuart, McGann and Dewey, 1945), and the third was on washed cells equivalent to the concentrations found in the whole medium. The samples to be tested were added to the *L. casei* base medium and sterilized by auto-

claving. The results of these experiments are given in Table IV. Appreciable amounts of riboflavin, pantothen, and biotin are synthesized by the ciliates. Increases in amounts of niacin are so small that they probably lack significance and there appears to be no increase in pyridoxine. It must be remembered, however, that the growth in the niacin-free medium is less at 72 hours than in the riboflavin-, pantothen-, or biotin-free media, while the maximum population reached in the pyridoxine-free medium never exceeded 20,000 ciliates per ml. The amounts of the vitamins detected represent minimums, as no attempt was made to release any which may have been bound (except by autoclaving). It is to be noted that all vitamins which were synthesized remained in the cells. This was also found to be true in the case of the biosynthesis of thiamine by *T. geleii* W (Kidder and Dewey, 1942).

### DISCUSSION

Due to the various treatments necessary for the removal of vitamins none of the Factor II preparations used in this study produced as high yields as had been previously obtained (Kidder and Dewey, 1945d; Kidder, 1945). While the riboflavin-free preparation was essentially the same as had been used for the study of purines and pyrimidines and of folic acid, variations in potency of Factor II activity were evident. This is due almost entirely to the degree of adsorption on the activated charcoal. Slight variations of temperature appear to effect the degree to which Factor II is lost, so that a critical balance is found between the complete removal of the vitamins and the loss of Factor II activity. In this study the emphasis was placed on the vitamin removal at a sacrifice of yield.

The findings of Hall and Cosgrove (1944) on the importance of riboflavin for their strain of *Tetrahymena geleii* does not seem inconsistent with the present observations. They state that heat—and alkali-treated casein did not support growth unless supplemented with thiamine, and even then poorly. The addition of riboflavin together with the thiamine, however, permitted as good growth as did the casein medium before heating. There can be no doubt as to the stimulatory effect of riboflavin, and it is altogether possible that it may function as a detoxifying agent as well. The detoxifying action of thiamine has been suggested previously in this connection (Kidder and Dewey, 1944).

In addition to the vitamins which have already been investigated for *T. geleii* W there remain at least three of the commonly recognized ones about which little is known. These are *p*-aminobenzoic acid, inositol and choline. As yet we have not had the opportunity to test for the last two, but preliminary work has been started on the first. The commonly employed technique of adding sulfonamides to the medium has indicated that this ciliate requires excessive amounts of the inhibitor to effect growth. The inhibition to growth at these high levels is not completely reversed with *p*-aminobenzoic acid, and the evidence indicates that purines are also involved. This study awaits completion and will be reported at a later date, but it appears that *T. geleii* W may be independent of an exogenous supply of *p*-aminobenzoic acid.

The only other protozoan of animal nature about which there appears to be critical data regarding the requirements of the vitamins studied here is *Colpoda duodenaria* (Tatum, Garnjobst, and Taylor, 1942; Garnjobst, Tatum and Taylor, 1943).

Colpoda requires large amounts of thiamine, pantothen, riboflavin, nicotinamide, and pyridoxine. It does not require *p*-aminobenzoic acid, biotin, or inositol, while the status of choline and folic acid is still unknown. Moreover, Colpoda was shown (Garnjobst, Tatum, and Taylor, 1943) by the *Neurospora* test of Tatum and Beadle (1942) to either release bound biotin from the bacterial "plasmoptyzate" used or to synthesize this vitamin. This biotin appeared in the medium, however, and in this way differs from the condition found with *T. geleii* W where all of the vitamins arising by biosynthesis appear to bound in the cell protoplasm.

The biochemical investigations of *Tetrahymena geleii* W which have so far been completed permit a fairly complete view of its synthetic abilities. Added carbon sources appear to be unnecessary except as they may perform a sparing action on the amino acids. Inorganic salts certainly are essential (Hall and Cosgrove, 1944; Kidder and Dewey, 1944) although the question of which elements need to be included is yet to be determined. The commonly employed inorganic salts usually accepted as being physiologically important satisfy the ciliate requirements. Nine amino acids are to be classed as indispensable for this strain (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine) while arginine is synthesized at so low a rate that its inclusion becomes obligatory. Serine is extremely stimulatory, but its place may be taken by others of the dispensable amino acids (Kidder and Dewey, 1945c). The list of essential growth factors for this strain is not long. Purines (most effectively guanine) and pyrimidines (cytidylic acid and/or uracil) must be supplied in rather large amounts (Kidder and Dewey, 1945d), and folic acid must be present in amounts in excess of that required for most of the folic acid-requiring bacteria (Kidder, 1945). Factor II must be supplied. This substance (or substances) is still chemically undefined, but it possesses similarities to the "streptogenin" of Woolley (1941) and Sprince and Woolley (1944).

Biosynthesis of riboflavin, pantothen, and biotin can be accomplished by *T. geleii* W. Indefinitely transplantable growth results without exogenous thiamine (Kidder and Dewey, 1942; 1944; 1945b), riboflavin, pantothen, biotin, niacin, or pyridoxine. There is some evidence to indicate that *p*-aminobenzoic acid may not be essential, and the status of inositol and choline is still unknown.

For practical purposes it is always of advantage to include any substances of a stimulatory nature. The absence of any one of the stimulatory substances (thiamine, riboflavin, pantothen, biotin, niacin, pyridoxine) will become a limiting factor, decreasing the growth rate or the maximum yield or the longevity of the culture (Johnson and Baker, 1942; Hall, 1944). The stimulatory vitamins should be included in the culture medium of this ciliate when maximum growth is desired.

#### SUMMARY

1. It has been possible to prepare media for the growth of *Tetrahymena geleii* W which are free of riboflavin, pantothen, biotin, niacin, and pyridoxine, as determined by the *Lactobacillus casei* test.

2. *T. geleii* W is not dependent on an exogenous source of any one of the above vitamins. Omission of any one, however, reduces the maximum yield and, with the single exception of pantothen, the growth rate.

3. Biosynthesis of appreciable amounts of riboflavin, pantothen, and biotin occurs. These vitamins are found bound in the cell protoplasm. No significant increases of pyridoxine by biosynthesis were found.

4. The five vitamins listed are not essential growth factors for *T. geleii* W but are stimulatory factors, and as such should be included in the medium for optimum growth.

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# THE STRUCTURE OF MEIOTIC CHROMOSOMES IN THE GRASSHOPPER AND ITS BEARING ON THE NATURE OF "CHROMOMERES" AND "LAMP-BRUSH CHROMOSOMES"

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The nature of the gene is one of the fundamental problems in modern biology. Since the genes are located in the chromosomes, the structure, chemistry, and metabolism of the chromosomes are of special significance for the understanding of the gene and gene action. The prevalent interpretation of chromosome structure has developed as a kind of compromise between two originally opposed views, the "chromomere hypothesis" of Balbiani, Pfitzner, and Strasburger and the "chromonema hypothesis" of Baranetzky, Bonnevie, and Vejdovsky.<sup>2</sup> According to the "chromomere hypothesis," the chromosome consists of a series of small beads or discs strung together. During prophase they approach each other, fuse into larger complexes, and finally disappear in the thick rod-shaped metaphase chromosomes. For the "chromonema hypothesis" on the other hand, the fundamental unit of the chromosome is a coiled thread, tightly wound in a helix at metaphase and more or less uncoiled during interphase. Both chromomeres and spirals were discovered about the same time (Balbiani, 1876; Pfitzner, 1882; Baranetzky, 1880). Yet more and more structures first described as "chromomeres" have turned out to be coils and today the "chromomere" is in full retreat into the sub-microscopic level. Strasburger's "chromomeres" in *Tradescantia* pollen mother cells had been clearly shown to be spirals by Baranetzky (1880); Pfitzner's "granules" in somatic prophases of the salamander were resolved into coils by Schneider (1910) and by Lee (1921), who concluded that all "chromomeres" are in reality turns in the helix. The modern view which is accepted by most cytologists today and is based mainly on Heitz (1935), holds that the true "chromomeres" (Belling's ultimate chromomeres) can only be seen in the prophase of meiosis (leptotene) and in the curious giant chromosomes of dipteran larvae, where the chromonemata are assumed to be completely uncoiled. According to this view (Reuter, 1930; Heitz, 1935; Darlington, 1937; White, 1937; Geitler, 1938; Koltzoff, 1938; Kuwada, 1939; Nebel, 1939; Huskins, 1941, 1942; Straub, 1943) the chromonema consists of chromomeres of different but constant size, rich in nucleic acid, connected by protein fibrils. The chromomeres bear the genes, they reproduce as specific units and they synapse in meiotic prophase. They are the visible expression of the linear arrangement of the genes.

<sup>1</sup> Part of the work for this paper was done in the Department of Biology, Johns Hopkins University.

<sup>2</sup> The "vacuolization hypothesis" of Grégoire and his school, denying both chromomeres and chromonemata, has been thoroughly disproved by the work of the last twenty years and need not be discussed here.

Yet even in leptotene chromosomes the "chromomeres" were found to be coils by several authors. They were first described as such in *Tradescantia* by Kaufmann (1931), who nevertheless accepted the "chromomere" interpretation for other plants and animals (Kaufmann, 1936). Koshy (1934, 1937) found the leptotene chromosome to be coiled in *Allium* and *Aloe*, Naithani (1937) in *Hyacinthus*. Smith (1932) suggested that the beadlike appearance of the leptotene in *Galtonia* might be due to twists in the chromonema and Hoare (1934) noted that the zygotene threads give the impression of two tightly coiled chromonemata. Kuwada (1939) pointed out that sharp turns in the coils might easily be mistaken for "chromomeres." In *Tradescantia*, Swanson (1943) found no "chromomeres" which could not be resolved into coils, and he suggested that a chromomere pattern such as that in maize might be due to differential spiralization.

Yet most recent discussions on the gene and chromosome structure cling tenaciously to the belief that "chromomeres" are real (e.g., Schultz, 1944). The main evidence usually presented, besides the salivary chromosomes of dipteran larvae, is the observations of Wenrich (1916), Lewis and Robertson (1916), and Chambers (1924) on the large chromosomes in grasshopper spermatocytes. To re-examine this evidence is the purpose of the present investigation.

#### MATERIAL AND METHODS

Spermatocytes of *Chorthippus curtipennis*, *Chorthophaga viridifasciata*, *Dissosteira carolina*, *Melanoplus femur-rubrum*, *Arphia* sp., *Hippiscus* sp., and *Orphulella* sp. were studied in sections (fixation: B 15 and Sanfelice, stain: Feulgen), and aceto-orcein smears. For the detailed study of leptotene chromosomes sections stained with Feulgen were found to be more reliable than smears. To uncoil chromosomes, testes were submersed for one-two hours in  $2 \cdot 10^{-3}$  M KCN in Bělař solution (Bělař, 1929) before smearing (Oura, 1936). The optics used consisted of a Zeiss applanatic condenser N.A. 1.4, Zeiss 2 mm. objective N.A. 1.4 and  $15\times$  ocular. The photographs (except Figure 12) were taken with the same optics and a Bausch and Lomb photomicrographic camera type H. The stereoscopic photographs were made by shifting the substage diaphragm maximally to the left and right respectively for the two exposures.<sup>8</sup>

#### THE STRUCTURE OF LEPTOTENE CHROMOSOMES

On casual examination the slender, irregularly twisted chromosomes at leptotene have a beaded appearance as has been so often described in the literature (for a review see Reuter, 1930). A detailed study with the best optics and a delicate use of the fine adjustment screw of the microscope, however, resolves the beads or "chromomeres" into turns of a narrowly pitched coil<sup>4</sup> (Figures 1, 6a, and 13). With Feulgen the chromosome stains evenly throughout its length and there are no Feulgen-negative "interchromomeric fibrils." This uniform nature of the

<sup>8</sup> I wish to thank Mr. John Spurbeck, Dept. of Biology, Johns Hopkins University, for help with the photomicrographs.

<sup>4</sup> Mr. L. Vanderlyn, Dept. of Zoology, University of Pennsylvania, informs me that he has come independently to the conclusion that the "chromomeres" are in reality gyres in the chromonemata. In a forthcoming paper he will trace the origin of these from the unpacking coils of the preleptotene in *Podisma alpina*.

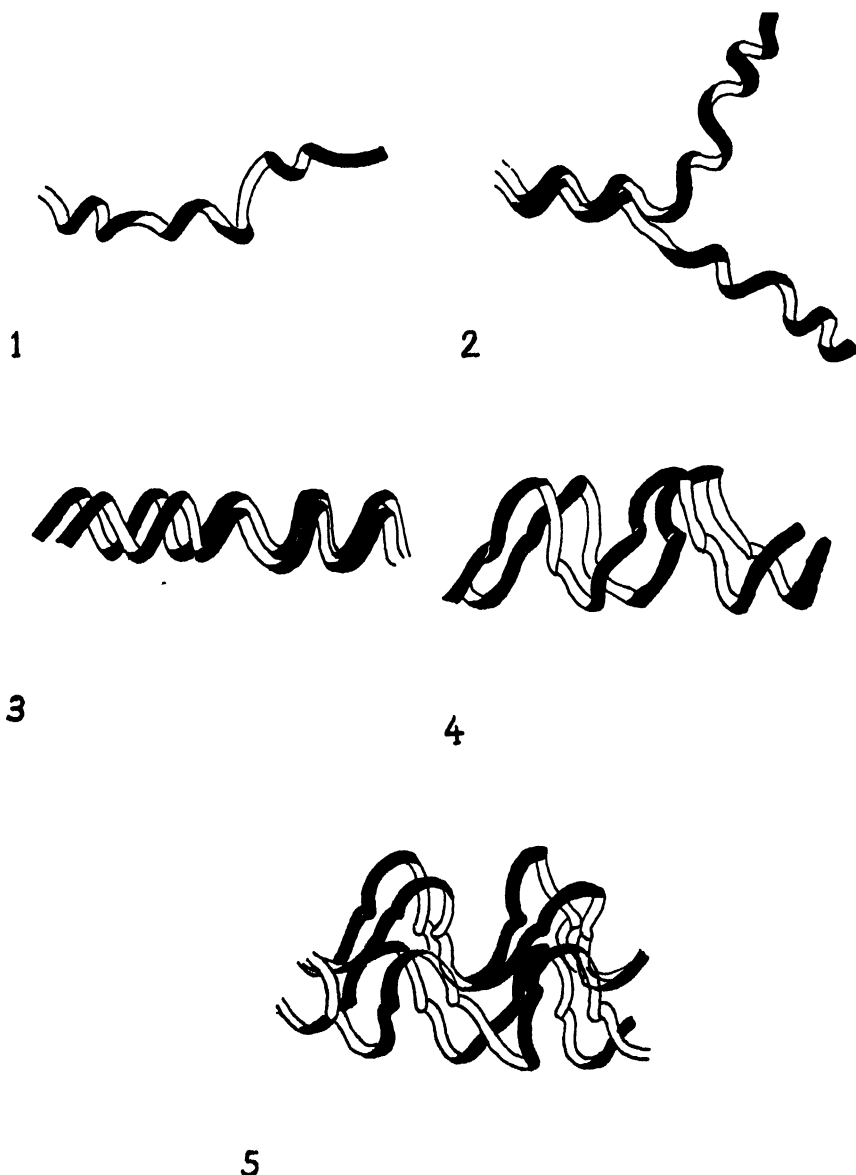
leptotene chromosomes can best be seen in well fixed sections. A chromosome, followed with the fine adjustment as it winds itself through the nucleus, is seen to be a thread of uniform thickness thrown into a tight, irregular helix. The narrow turns of this coil where the chromosome overlaps itself, appear as "chromomeres." The gyres can vary in width and may be unevenly spaced (see Figure 13). This can give the impression of different sized chromomeres. The width of the thread and the tightness of the helix are characteristic for each species of grasshopper studied. In aceto-orcein smears, when the chromosome has been under shear or pressure, an apparent chromomeric structure is more pronounced. This is due to the wax-like consistency of the chromosome which causes its gyres to fuse or be pulled out and otherwise distorted. Chromosomes, in which the coils can be clearly seen, can easily be transformed into the classical string of beads simply by exerting pressure on the coverslip and smearing them out. It is interesting to note in this connection that Belling (1931) emphasized that chromomeres are not clear in sections and that one has to use smears to make them visible.

When does that tight irregular coil of the leptotene chromosome originate? Is there any stage when the chromonemata are completely stretched out and without any signs of coiling? In all the grasshoppers studied no chromosome was found that did not show some degree of coiling. Furthermore, the characteristic coil of the leptotene chromosome is already present in the interphase and unravelling stage of preleptotene. We must assume that the leptotene spiral originates in the interphase or telophase of the preceding division. This origin of a prophase helix in the preceding telophase has been demonstrated by Sparrow (1942) in the microspore division in *Tradescantia*. The chromosome of the unravelling stage is thus doubly coiled (Figure 7). It shows the wide gyres of the previous metaphase relaxing into the relic coils of leptotene and the small tight helix which is destined to enlarge during pachytene and become the major coil of the first meiotic metaphase chromosome. This structure of the preleptotene chromosome was indicated clearly in McClung's figures for *Mecostethus lineatus* (esp. Figure 43, McClung, 1927). The heteropycnotic X chromosome in the prophase of grasshopper spermatocytes, which does not unwind in preleptotene and is thus comparable to the preleptotene autosomes in structure, similarly discloses a small tight helix and a wide irregular coil as Coleman (1943) has demonstrated.

Since the preleptotene chromosome consists of at least two chromonemata the leptotene chromosome also must be double (Robertson, 1931). The split between the chromatids can sometimes be discerned, especially in the turns of the coil, but usually the sister strands are closely appressed. They seem to form a plectonemic spiral, though this could not be determined with certainty.

#### THE STRUCTURE OF ZYGOTENE CHROMOSOMES

The pairing of homologous chromosomes at zygotene thus takes place between two coiled structures. The gyres of the two chromosomes fit into each other and become more or less closely appressed (Figures 2 and 6b). The bivalent now forms a paranemic coil. Just as the gyres in leptotene were mistaken for "chromomeres," so the gyres of the parallel coil in the bivalent were thought to be paired "chromomeres."



FIGURES 1-5. Diagrammatic representation of chromosome structure during meiotic prophase of the grasshopper.

FIGURE 1. Leptotene.

FIGURE 2. Zygotene.

FIGURE 3. Pachytene. The homologues can be either slightly separated or closely appressed.

FIGURE 4. Later pachytene. Appearance of the minor coil.

FIGURE 5. Diplotene. The chromonemata have separated laterally. This represents in essence also the structure of "lamp-brush chromosomes."

## THE STRUCTURE OF PACHYTENE CHROMOSOMES

During pachytene the helices of the paired chromosomes increase in width and the number of gyres decreases. This process is identical to that described by Swanson (1942a) for *Tradescantia* (despiralization cycle). If the chromosomes are closely appressed only one helix is visible. When the coils separate slightly a reticular or vacuolated appearance is produced, though often two parallel helices can be clearly discerned (Figures 3 and 8). In late pachytene an irregular waviness appears on the gyres of the pachytene coil; this sometimes looks like a very fine spiral of narrow pitch. It most likely corresponds to the minor spiral described in plant chromosomes (Figures 4 and 9).

## THE STRUCTURE OF THE CHROMOSOMES DURING DIPLOTENE AND DIAKINESIS

In this stage the chromosomes are most difficult to analyze. They are usually described in the literature as diffuse, having fuzzy or woolly fringes (see for instance Nebel and Ruttle, 1937). The better the general fixation seems to be, the less distinct or sharp the chromosomes appear. However, after submersing the cells for one to two hours in  $2 \cdot 10^{-3}$  M KCN in Bělař solution and staining in aceto-orcein, the structure of the diakinesis chromosome and the reason for its woolly appearance becomes quite clear. The lateral separation of the chromonemata which had already begun in pachytene has progressed much further, so that their gyres now overlap only within a narrow central region. This region appears as a beaded darker core of the chromosome. The gyres of the major coil of the chromonemata form loops projecting beyond this central core (Figures 5 and 14). It is these loops of the individual chromonemata which give the chromosome its hairy appearance. If the separation of the coiled threads is great the chromosome looks like a dark, beaded rod with loops or hairs at regular intervals (Figure 14a). When the lateral shifting is less the chromosome gives the impression of a double beaded rod, the loops or hairs now of course being shorter (Figure 14b). These appearances can easily be explained on a model of four simultaneously coiled wires. Sometimes one or more irregular turns of the minor coil can be seen on the loops.

In this stage there is further evidence against the reality of "chromomeres." If the apparent thickenings in the leptotene chromosome were constant units of definite size, they should be visible also in the loops of the diplotene chromatids.

## PLATE I

FIGURE 6. *Chorthophaga*, zygotene. Pretreated with ammonia vapor. Aceto-orcein smear. Note the coil of the univalent at *a* and the paranemic helix of the bivalent at *b*.

FIGURE 7. *Chorthophaga*, preleptotene. Aceto-orcein smear. Irregular "major coil" in the process of unravelling. The narrowly pitched helix ("minor coil") corresponds to the leptotene spiral (arrows).

FIGURE 8. *Chorthippus*, early pachytene. Section. Fixed with Sanfelice and stained with Feulgen.

FIGURE 9. *Hippiscus*, late pachytene. Section. Fixed with Sanfelice and stained with Feulgen.

FIGURES 10 AND 11. *Orphulella*, pachytene. Pretreated for 2 hours in KCN. Aceto-orcein smear. The heterochromatic knobs have been resolved into coils (arrows).

FIGURE 12. Fragment of a "lamp-brush chromosome" from a frog oöcyte. Aceto-orcein smear. Note the loops of the major coil and the minor coil (arrows). Zeiss 3 mm. objective, 15× ocular.

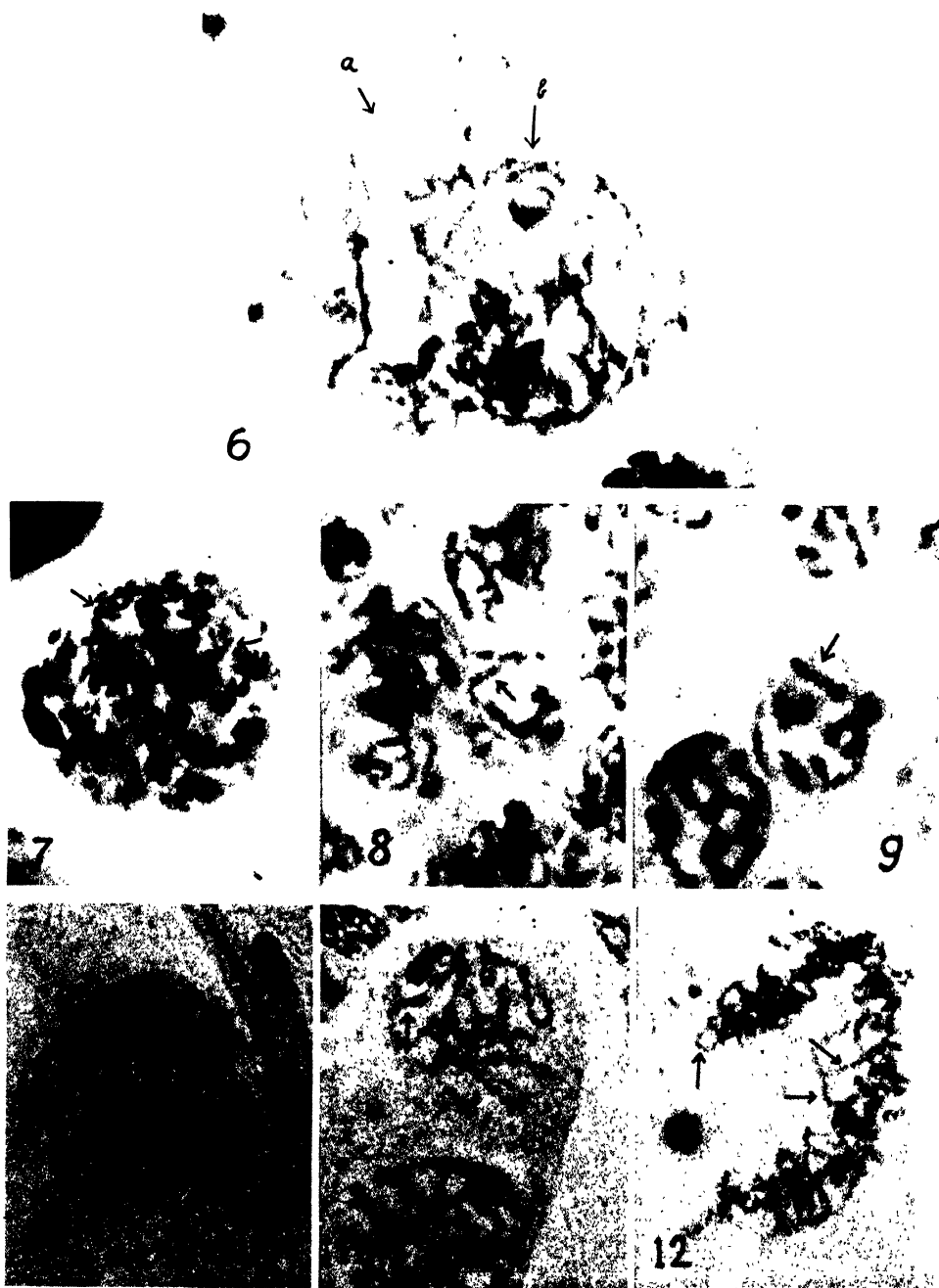


PLATE I

These chromatids, however, never show any beaded structure. The despiralization already noted in pachytene has continued and has resulted in an increase in width and decrease in the number of gyres with a consequent shortening and thickening of the chromosome.

### THE STRUCTURE OF METAPHASE CHROMOSOMES

At the end of diakinesis the gyres of the chromatids become more closely spaced along the chromosome axis, leading to a further shortening of the chromosome and a fusion of the "chromatic coating" (Ris, 1942) of the individual chromatids, so that a uniformly staining body results. The chromatids retain their lateral separation, causing what is sometimes observed as a reticulate or vacuolated appearance of the metaphase chromosomes.

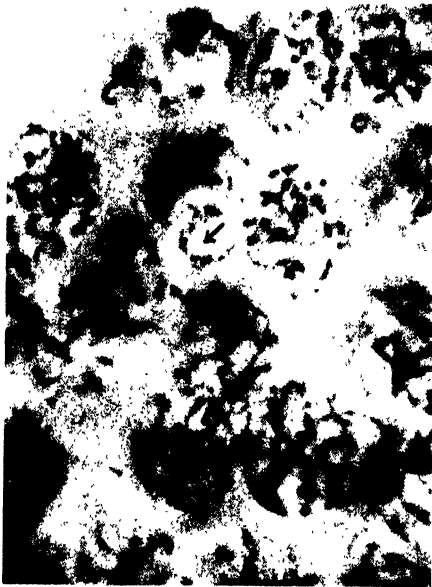
### THE NATURE OF THE HETEROPYCNOTIC REGIONS IN ORPHULELLA

During meiotic prophase the chromosomes of *Orphulella* carry small, knob-like, darkly staining bodies, especially at their ends. These structures resemble the large "chromomeres" described by Wenrich (1916) in *Phrynotettix*. Treatment with KCN for 3 hours causes a loosening of the chromosome helix and shows that these knobs are tightly coiled regions of the chromosome (Figures 10 and 11). It is evident that the different appearance of such heteropycnotic regions in meiotic chromosomes is mainly due to differential coiling of the chromonemata as has been shown for the X chromosome by Coleman (1943). Similarly Wilson and Boothroyd (1944) have demonstrated that heterochromatic differentiations after cold treatment are the result of differential coiling.

### DISCUSSION

#### *Chromomeres*

The synthesis of cytology and genetics in the chromosome theory of inheritance has had a stimulating effect on the investigation of chromosomes. Yet the knowledge of the intimate structure of the chromosome has been retarded rather than furthered by the influence of genetics. The constant desire to find visual expression of the linear order of genes has led to the perpetuation of misinterpretations of the microscopic image. Indeed cytogenetics has established beyond doubt the longitudinal differentiation of chromosomes, but it is not justifiable to conclude that the units of this differentiation are microscopically visible particles. Thus observations which did not agree with the "chromomere" hypothesis tended to be ignored. The extensive literature on the subject (see Reuter, 1930) shows the widespread acceptance as well as the great versatility of the chromomere concept. Almost any expression of unevenness along the chromosome was at one time or other called "chromomere." The first pictures of "chromomeres" were published by Balbiani (1876) and Pfitzner (1882). Both described prophase and metaphase chromosomes in somatic cells. Today there can be no doubt that they saw the gyres of the somatic helix (Schneider, 1910; Lee, 1921; Creighton, 1938). Strasburger (1882) and Farmer and Shove (1905) described disc-like "chromomeres" in meiotic metaphase chromosomes of *Tradescantia*. We know now that they mistook the gyres of the major coil for discs. Quite often chromocenters in



13



14



## PLATE II

FIGURE 13. Stereophotomicrograph, *Chorthippus* leptotene. Section. Fixed with Sanfelice and stained with Feulgen and Iron hematoxylin. Note the coiled leptotene chromosomes (arrow).

FIGURE 14. Stereophotomicrograph, *Hippiscus* diakinesis. Pretreated with KCN. Aceto-orcein smear. Note the loops of the major coil which give the chromosomes at this stage the fuzzy appearance.

interphase nuclei and heteropycnotic regions on the chromosome, such as found in the X chromosome of *Notonecta indica* (Browne, 1916), were called "chromomeres" (cf. Heitz, 1929). Shinke (1937) and Coleman (1940, 1941) have shown that such heteropycnotic regions are parts of the chromonema which remain tightly coiled or become precociously coiled. This could be confirmed in the present paper for the "knobs" of the meiotic chromosomes of *Orphulella*. Thus, one more "chromomere" was reduced to chromonematic coiling. There remained the "ultimate chromomere" of Belling (1928), the only bona fide "chromomere" according to most modern cytologists. This "chromomere" can only be seen in meiotic prophase and in salivary chromosomes of dipteran larvae, where the chromonemata are assumed to be maximally stretched. Let us examine point for point the evidence which is given for the reality of these "chromomeres" (see reviews cited in introduction).

(a) "*The chromomeres are seen in living cells and cannot be artefacts.*" Bělář (1928) described "chromomeres" in living spermatocytes of the grasshopper. An analysis of his figure shows that he did not see chromomeres but the coils of diakinesis chromosomes. Lewis and Robertson (1916) and Chambers (1924) found "chromomeres" in the leptotene of living grasshopper spermatocytes. This may show that the structures observed are not fixation artefacts, but it certainly is easier to misinterpret narrow coils as granules in unstained cells where the chromosomes are hardly visible, than in well stained preparations. Yet there is a very interesting observation by Chambers (1924, page 270) which seems to have been overlooked by himself as well as most reviewers of chromosome structure. He writes: "If one of the early prophase chromosomes with ragged granular outlines be seized with a needle and rapidly pulled across the field so as to stretch it, the granules disappear and the whole substance becomes homogeneous." So Chamber's microdissection study does not support the "chromomere" hypothesis, but rather the assumption of a uniform but coiled leptotene chromosome.

(b) "*The chromomeres have specific and constant sizes and form a definite pattern.*" The classical examples are *Dendrocoelum* (Gelei, 1921) and *Phrynotettix* (Wenrich, 1916). The observed patterns in these and other forms are an expression of the longitudinal differentiation of the chromosome. This differentiation is real. But the nature of this differentiation now turns out to be differential coiling and not a sequence of discrete bodies of different sizes. The large "chromomeres" in *Phrynotettix* are heterochromatic regions along the chromosome similar to those found in certain plant chromosomes and those described for *Orphulella* in this paper. In *Veltheimia viridifolia* Coleman (1940) could show that such heterochromatic regions are closely coiled sections of the chromonema. They correspond in structure to the differential segment in *Rhoeo* (Coleman, 1941) and the chromocenters in various animals and plants (Shinke, 1937). The knobs in maize are most probably of a similar nature.

(c) "*The chromomeres of homologous chromosomes pair specifically at zygotene.*" Just as the turns in the spiral give the impression of "chromomeres" at leptotene, the paranemic spiral of the paired bivalent simulates a row of paired granules. Since homologous regions of the chromosomes pair, it is evident that heterochromatic sections will come to lie side by side in the pachytene chromosomes.

(d) "*The number of chromomeres in leptotene corresponds approximately to the number of genes in Lilium* (Belling, 1928). *In salivary chromosomes the*

*bands, which correspond to the leptotene chromomeres, were shown to be closely associated with certain genes (Muller and Prokofyeva, 1935)."*

Belling's estimate of the number of genes in *Lilium* was entirely arbitrary and he had no direct evidence for a correlation of "ultimate chromomeres" and genes. In salivary chromosomes of *Drosophila*, however, a great number of workers have proven beyond doubt that the visible "bands" are correlated with certain genes. A recent analysis of the salivary chromosomes of *Sciara* in collaboration with Dr. Helen Crouse (in press) has shown that the "granules" and "bands" are misinterpretations of a very complicated spiralization of a bundle of chromonemata. What has been described as a "chromomere" corresponding to a gene represents in reality a region of relatively considerable length along the chromonema. The cytogenetic work on *Drosophila* salivary chromosomes is not evidence for a "chromomeric" structure of the chromonema, but shows that certain sections of the uniform chromonematic thread correspond to definite genes and that the detailed nature of the coiling in these interphase chromosomes is closely correlated with a genetic specificity on a submicroscopic level.

In summary this is the evidence against the existence of "chromomeres": (a) In living cells the microdissection experiment of Chambers (1924) shows that the leptotene chromosome can be stretched into a uniform thread. (b) In several plants such as *Tradescantia* (Kaufmann, 1931; Swanson, 1943), *Allium* and *Aloe* (Koshy, 1934, 1937), *Hyacinthus* (Naithani, 1937), and in the grasshopper the leptotene chromosome consists of a uniform, coiled thread, Feulgen-positive throughout its length. No evidence of interchromomeric fibrils can be found. The leptotene coils can be followed into the pachytene where they increase in width and decrease in number. This explains the observation of many authors (e.g., Belling, 1931) that the "chromomeres" increase in size and decrease in number during the course of prophase. (c) In the diplotene chromosomes of the grasshopper no "chromomeres" can be seen in the large loops of the chromatids. If specific "chromomeric" granules were present at leptotene they should be visible also in the chromonema of diplotene. (d) McClintock (1944) has shown in maize that at least one gene is located in the interchromomeric thread between the terminal knob and the first "chromomere" on chromosome nine. This disproves definitely the idea, at least for maize, that the genes are necessarily located in the "chromomeres" which are connected by non-genic fibrils.

#### *Diplotene chromosomes and "lamp-brush chromosomes"*

The coiling cycle in the grasshopper appears to be identical with that described by Swanson (1942, 1943) for *Tradescantia*. The leptotene coil develops into the major coil of diakinesis and metaphase through despiralization. There is no definite minor coil, but from late pachytene on, an irregular waviness appears on the loops of the chromatids, resembling an incipient helix. A minor coil was seen in spermatocytes of another orthopteran, *Podisma*, by Makino (1936). In *Trillium* (Huskins, 1941) there seems to be a similar waviness instead of a definite helix as was demonstrated for *Tradescantia*. This difference in the appearance of the minor coil seems to be mainly one of timing of the spiralization cycle as Kuwada (1938) has suggested. In the grasshopper the chromatids have never been seen completely separated in diakinesis or metaphase. Their coils sometimes appear

interlocked as Kuwada (1938) found in *Tradescantia*, but this could not be definitely determined. Swanson (1942b) has shown that the terminalization of chiasmata is correlated with the despiralization of the major coil in *Tradescantia*. The same process takes place in the grasshopper and it is most likely that here, too, terminalization of chiasmata is the consequence of despiralization of the major coil.

The diffuse appearance of orthopteran as well as most other animal chromosomes in diplotene has made their analysis rather difficult. The chromonema is generally of smaller diameter than in plant chromosomes and therefore the delicate loops of the major coils escaped observation. This diffuse structure is due to a lateral separation of the chromatids in contrast to the usual appression of the chromatids in plant chromosomes. Under certain conditions, and especially in diakinesis, plant chromosomes also show a separation of chromatids. They then give the same pictures as diplotene chromosomes of animals (see the anaphase chromosome of desynaptic *Trillium* in Figure 9 of Sparrow, Huskins and Wilson, 1941; Swanson, 1942a, 1943, and Kuwada and Nakamura, 1938 for *Tradescantia*). Plant and animal chromosomes have often been described as reticulate or vacuolated. Grégoire and his school based on this their "vacuolization hypothesis" of chromosome structure. All their pictures can today be explained on the simple assumption of a multiple stranded helix with the chromonemata more or less appressed or opened up.

When the lateral separation of the chromonemata is great and the loops only faintly stained, the chromosome may appear covered with a layer of achromatic material (often described as "matrix" or "sheath"; see for instance Lee, 1921 and McClung, 1941, Figure 7). Probably many a "matrix" in the literature is nothing but the apparant connection between faintly staining outer loops, running at an even distance from the darker core of the chromosome where the chromonemata overlap. Makino (1936) published some photographs of diakinesis and metaphase chromosomes of *Podisma* which at first seem to contradict my interpretation of these stages. He shows a dark inner coil sometimes appearing double, surrounded by a light "matrix." Faint strands are sometimes seen to connect the central spiral with the border of the "matrix." Yet it is very easy to understand these figures with the help of a model of four wires coiled together. When two are maximally separated laterally and two stay appressed in the center, Makino's coil and matrix become explainable. The outer coils are not at all or only faintly stained in his gentian violet preparations and their outer boundary suggests the presence of a "matrix."

The previous studies of diplotene chromosomes of Orthoptera have completely ignored these outer gyres of the chromonemata. They were described as woolly threads or brushlike projections on the surface of the chromosome, but not as an essential part of it. Thus the pictures of Hearne and Huskins (1934), Nebel and Ruttle (1937), Darlington (1936), and the McClung school are based on optical illusions or too light staining. What were described as "chromomeres" in this stage are the points of overlap of the chromonemata. Darlington (1936) has studied relational coiling of chromatids and chromosomes in pachytene and diplotene. What he pictured as one single chromatid, however, is not a continuous structure, but a series of nodes of separate overlapping major coils. His relational coil of chromatids is therefore an optical illusion. Only a complete stretching of

the major coil could reveal whether the chromatids are wound around each other (see Kuwada, 1938).

Many oöcytes and spermatocytes in diplotene undergo a so-called "diffuse stage," which is correlated with the growth of the cell. The chromosomes stain only faintly and lose their definite shapes; they may even disappear into a reticular structure. In the grasshopper the diffuse nature of the chromosomes is due to the loosening and separation of the individual chromonemata of the major coil. This more or less pronounced loosening up of the gyres, combined possibly with some chemical changes in the composition of the chromatin, can explain the appearance of diplotene chromosomes during this stage in spermatocytes and oöcytes.

The diplotene chromosomes in the large oocytes of some vertebrates have particularly interested the cytologist ever since their discovery by Rückert in 1892, because of their tremendous size. Their fuzzy and brush-like appearance warranted the name "lamp-brush chromosomes." Duryee (1937, 1938, 1939, 1941) has recently studied these chromosomes in great detail in the frog and salamander, and concludes that (1) they represent paired gelatinous cylinders in which the chromomeres are embedded. (2) From these chromomeres lateral loops grow out. He likens this growth to that of a crystal or the reproduction of a virus. (3) In a later stage, before the maturation divisions take place, these lateral loops are thrown off into the cytoplasm as genic products essential for the early embryo.

Painter (1940) came to somewhat different conclusions. He considers "lamp-brush chromosomes" to be chromosome aggregates, which originated through endomitosis and the loops to correspond to whole chromosomes. Material from thousands of such chromosomes, he maintains, is thrown into the cytoplasm as substrate for the synthesis of cleavage chromosomes. Koltzoff (1938) thinks that the lateral projections are side branches of the chromomeres which then are given off into the cytoplasm.

In contrast to Duryee, Koltzoff, and Painter, it is here suggested that "lamp-brush chromosomes" are typical diplotene chromosomes which differ from other diplotene chromosomes only in the tremendous longitudinal growth of the chromonemata. The loops are then the major coils of the laterally separated chromonemata, the "chromomeres" are simply overlaps of the strands just as in diplotene chromosomes of the grasshopper. Figure 12 shows a fragment of a "lamp-brush chromosome" of a frog oöcyte, smeared in aceto-orcein. The somewhat distorted large loops of the major coil and the minor coil are easily visible.

The evidence for this interpretation may be summarized as follows: (a) The loops are continuous as Rückert (1892) has already observed. He followed the chromonema for several turns. He also pointed out that the granules ("chromomeres") are not real, but optical sections of the overlapping threads. The denser inner region of the chromosome he described as due to the radial arrangement of the threads. (b) "Lamp-brush chromosomes" are diplotene chromosomes and except for their greater size have the same appearance as the diplotene chromosome of the grasshopper. Since it has been shown here that the loops are simply the gyres of the major coil of the separate chromonemata, one can conclude that the corresponding appearance of the "lamp-brush chromosome" is the result of a similar structure. (c) Koltzoff (1938) has published drawings of cross sections of "lamp-brush chromosomes" (his Figure 10, b and c). These cross sections look like a star with characteristically eight rays. These eight rays are most likely the eight

half-chromatids which form independent loops, though Koltzoff saw them as brush-like projections.

The reduction in chromosome size just before the meiotic divisions is accomplished then not by throwing off parts of the chromosome or entire chromosomes, but by elimination of material on a submicroscopic level.

### *The microscopic organization of chromosomes*

Kuwada (1939) in his review of chromosome structure predicted that the spiral theory might well prove capable of harmonizing the various hypotheses of chromosome structure. Such a uniform interpretation of the structure of all types of chromosomes is now possible. The unit of the chromosome is the chromonema, a microscopically uniform thread. This chromonema is never completely straightened out, but always shows some degree of spiralization. This coiling is not at random, but, as the salivary chromosomes and heterochromatic regions show, is an expression of the longitudinal differentiation of the chromonema and closely correlated with the genes. It is, in other words, an expression of submicroscopic structure and possibly the functional state of the gene (cf. heterochromatin). The microscopic uniformity of course does not exclude a great variability of submicroscopic structure and chemical composition along the chromonema. During the mitotic cycle there develops a condensed chromosome through despiralization of the incipient coil of early prophase. The differentiation of mitotic chromosomes, primary and secondary constrictions, satellites, and heterochromatic regions are expressions of the differential coiling of the chromonemata. In the resting nucleus of different tissues we often find different patterns of heterochromatin. It may be that differential spiralization of the chromonemata in resting cells is correlated with cell differentiation. The chromonema is not uniform in length, but it can vary greatly from cell to cell in the same organism, as well as in the same cell in different metabolic states. In many synthetically very active cells as for instance some oöcytes, nurse cells, gland cells (dipteran salivary glands), the total amount of chromatin is greatly increased. This is accomplished by an increase in the number of chromosomes (endomitosis, cf. Geitler, 1941), by a growth in length of the chromonemata (as in "lamp-brush chromosomes") or by both simultaneously (salivary chromosomes). In "lamp-brush" and salivary chromosomes the increase in length is tremendous and would be difficult to understand if only inert "genoplasm" or "matrix" (Koltzoff, 1938) had increased. More likely it is an increase in the volume of the gene complex, related to the greater metabolic activity. We have to look at the gene, therefore, not as a unit of constant and specific size as expressed in the "chromomere" hypothesis, but as a complex that is greatly variable in mass, depending on the metabolic activity of the nucleus.

### SUMMARY

1. "Chromomeres" do not exist as definite structures. What has been described as "chromomeres" are (a) misinterpretations of gyres of the chromonematic helix (leptotene, somatic prophase); (b) points of overlap of chromonemata (diplo-tene); (c) heterochromatic sections consisting of more tightly coiled regions of the chromonema. The fundamental unit of the chromosome is a microscopically

uniform thread. The longitudinal differentiation of the chromosome is due to differential coiling of this chromonema.

2. "Lamp-brush chromosomes" are typical diplotene chromosomes, but with tremendously elongated chromonemata. The side branches are the gyres of the major coils of the individual chromonemata, which have laterally separated from each other.

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